CALCIUM SIGNALING MECHANISMS MEDIATE CLOCK-CONTROLLED ATP GLIOTRANSMISSION AMONG IMMORTALIZED RAT SCN2.2 CELL CULTURES

A Thesis

by

JEFFREY FRANKLIN BURKEEN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2009

Major Subject: Biology

CALCIUM SIGNALING MECHANISMS MEDIATE CLOCK-CONTROLLED ATP GLIOTRANSMISSION AMONG IMMORTALIZED RAT SCN2.2 CELL CULTURES

A Thesis

by

JEFFREY FRANKLIN BURKEEN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Approved by:

Chair of Committee,	Mark J. Zoran
Committee Members,	David J. Earnest
	Robert C. Burghardt
Head of Department,	U.J. McMahan

August 2009

Major Subject: Biology

ABSTRACT

Calcium Signaling Mechanisms Mediate Clock-Controlled ATP Gliotransmission among Immortalized Rat SCN2.2 Cell Cultures. (August 2009)

Jeffrey Franklin Burkeen, B.S., University of Notre Dame

Chair of Advisory Committee: Dr. Mark J. Zoran

The hypothalamus is an integral part of the brain's regulation of mammalian physiology and behavior. Among many functions, this regulatory center activates the sympathetic nervous system, maintains appropriate body temperature, controls food intake, and controls release of hormones from the pituitary gland. Deep within the hypothalamus lie a paired cluster of cells, the suprachiasmatic nuclei (SCN), which function as the chief circadian pacemaker. The goal of the present thesis research was to study rhythmically controlled ATP gliotransmission. I used an immortalized SCN2.2 hypothalamic cell line to determine the mechanism by which ATP signaling is regulated in this context. Additionally, this research aimed to elucidate if clock-controlled ATP gliotransmission is fundamentally distinct from stimulus-evoked calcium-dependent mechanisms that regulate intercellular ATP signaling among astrocytes.

In this thesis, I show that there are multiple ATP signaling mechanisms present among SCN2.2 cells. cAMP-dependent signaling mediates clock-controlled ATP accumulation but not stimulus-evoked ATP signaling. In addition, pharmacological

studies suggest that disparate purinergic receptor-mediated mechanisms are involved in the regulation of clock-controlled versus stimulus-evoked ATP signaling.

Rhythmic accumulation of ATP in SCN2.2 cultures is modulated by calcium-dependent processes. Peaks in ATP accumulation coincide with elevated mitochondrial calcium levels, while troughs in ATP accumulation coincide with periods of high cytosolic calcium levels, suggesting a possible mechanistic link between circadian shifts in intracellular calcium handling and ATP handling in SCN2.2 cells. Clock-controlled ATP accumulation in SCN2.2 cells is not a by-product of rhythmic cell cycle or rhythmic cell death.

Overall, my research suggests that the ATP accumulation rhythm in SCN2.2 cells is likely an output of the biological clock, mediated by astrocytic calcium signaling processes, and not an output of cell division or cell death. Estimation of ATP accumulation in SCN2.2 cultures at peak time points suggests that clock-controlled ATP release is critical to the function of astrocytes in the mammalian brain, perhaps in the regulation of brain metabolism, the regulation of sleep/wake physiology, or the integration of both.

ACKNOWLEDGEMENTS

I would like to thank many people for their guidance and support throughout my entire graduate research career and their contributions to the completion of this thesis. My committee chair, Dr. Mark Zoran, and my committee members, Dr. David Earnest and Dr. Robert Burghardt, have constantly given me direction and a resource to turn to in times of need. Without them by my side, this research would not have been possible.

I would also like to thank my Zoran lab colleagues and friends, Alisa Womac, Jarret Richardson, and Zane Lybrand for their encouragement and assistance.

TABLE OF CONTENTS

CHAPTER Page

LIST OF FIGURES

FIGURE Page

CHAPTER I

INTRODUCTION

The rotation of the earth around its axis generates daily environmental cycles. The daily change of greatest importance to organisms is the alternation of light and darkness. Many animal activities exhibit daily rhythmicity in synchrony with the 24 hour day, which consists of: light (day), a variable interval of darkness (night), and two twilights (dawn and dusk). This periodic succession of night and day has influenced life on earth for millions of years. In mammals, these periodic changes in the environment have been "internalized" in the form of an endogenous circadian clock. Its main function is to organize the time course of physiological, hormonal, and behavioral processes to enable the organism to anticipate these changing environmental conditions properly. The daily rhythms of behavior and physiology in mammals are generated and orchestrated from within a biological or circadian clock located in the anterior hypothalamus, the suprachiasmatic nuclei (SCN).

The Suprachiasmatic Nuclei

The SCN is the chief circadian pacemaker and biological clock in mammals. The location of this cellular clock within the hypothalamic SCN was discovered in the early 1970s (Moore and Eichler, 1972). Conclusive evidence that the SCN indeed

---

This thesis follows the style of The Journal of Neuroscience.

comprises the master circadian pacemaker came from combined lesion and transplantation studies. Transplantation of SCN tissue from mutant donor animals into SCN-lesioned wild-type hosts (or vice versa) conferred the circadian phenotype of the donor to the host (Ralph et al., 1990; Sujino et al., 2003). More recently, it became clear that the endogenous rhythm of the master oscillator is generated by a suite of clock genes, forming different sets of interlocking transcriptional/translational feedback loops (Kalsbeek et al., 2006). Despite this vast increase in recent knowledge, the link between the transcriptional and translational events of the molecular clock, on one hand, and the metabolic and electric activity of the SCN neurons (i.e., the output of the endogenous clock), on the other, is still not known. Moreover, the manner in which individual SCN neurons are assembled to create an integrated tissue pacemaker that can moderate the circadian behavior of an entire animal remains largely elusive (Kuhlman and McMahon, 2006).

The primary input pathway to the SCN is through retinal detection of light. Remarkably, the retinal photoreceptors that lead to visual image formation are not needed for circadian photoreception. Instead, a specialized population of intrinsically photosensitive retinal ganglion cells detects light and also projects directly to the SCN. In rodents, the retinohypothalamic tract (RHT) is composed primarily of the axons of these special ganglion cells, and the RHT is necessary for synchronization of the SCN clock to light.

The main output of the SCN is encoded by the neuronal firing rate of SCN neurons. In addition to direct synaptic interactions, rhythmic neuropeptide secretion into

the cerebrospinal fluid may be an important mechanism for regulation of downstream targets. Circadian rhythms in cellular metabolism, neuropeptide secretion, electrical activity, and gene expression are endogenous to the SCN *in vivo* and persist following isolation of SCN cells *in vitro* (Klein et al., 1991). Anatomically, the outputs of the SCN are focused within the hypothalamus, but their influence is widespread, consistent with the pervasive influence of the SCN on physiological functions ranging from the modulation of cognitive function to timing of neuroendocrine signaling.

The SCN is located directly above the optic chiasm in the anterior hypothalamus. It is organized into two compartments, the core and the shell. The core lies adjacent to the optic chiasm and comprises predominantly neurons producing vasoactive intestinal peptide (VIP) or gastrin-releasing peptide (GRP) co-localized with GABA and receives dense visual and midbrain raphe efferents (Moore et al., 2002). The shell surrounds the upper portion of the core and contains a large population of arginine vasopressin (AVP)-producing neurons in the dorsomedial portion, and a smaller population of calretinin (CAR)-producing neurons dorsally and laterally, colocalized with GABA. The shell receives input from non-visual cortical and subcortical regions.

SCN neurons from nocturnal and diurnal animals have shown synchronized circadian fluctuation in firing rate, with high firing rate occurring during subjective day and little to no firing activity occurring during subjective night (Gillette and Reppert, 1987) or high firing during midday in animals kept on an LD cycle (Yamazaki et al., 1998). The circadian rhythmicity in neuronal firing is controlled by the ‘clock’ found in each individual oscillator. The clock is comprised of several genes that participate in

transcriptional-translational feedback loops that activate and inhibit clock gene expression. These genes can also activate transcription for a number of clock-controlled genes, some of which can alter firing rate within individual neurons through transcriptional or posttranslational modification (Pennartz et al., 2002). Photic input from retinal ganglion cells activates the transcriptional-translational feedback loop in individual retinorecipient oscillators that are coupled somehow to produce 24-hour rhythms in firing.

The central model system of the following research is an immortalized cell line derived from the rat SCN (SCN2.2) which retains the endogenous oscillatory and pacemaking properties of the SCN *in vitro* and *in situ*. SCN 2.2 cells are capable of intrinsically generating self-sustained rhythms of gene expression and metabolism, of imposing these oscillations on cocultured fibroblasts, and of restoring behavioral rhythmicity when transplanted into SCN-lesioned hosts (Allen et al., 2001; Earnest et al., 1999). SCN2.2 cell cultures will be used in this research because of their significance to biological clock function, and also because of their emerging utility as a mammalian model system for the study of neural cell-cell signaling.

Astrocytic Cell-Cell Signaling

Cellular communication between neural cells has been well-researched and has highlighted the importance of glial cells in regulating synaptic transmission, leading to the proposal that synapses are tripartite (Araque et al., 1999). Chemical synapses are formed by the presynaptic neuron, from where neurotransmitters are released, the

postsynaptic neuron, which receives and integrates the information, and by a third component represented by astrocytic processes that tightly envelop neuronal elements. Because of this close anatomical relationship, astrocytes participate and/or regulate several brain functions including synaptic transmission, synaptogenesis and synaptic plasticity (Haydon, 2001). These glial cells express many ion channels, transporters, and receptors through which they can detect modifications in their environment. Following either agonist or synaptic stimulation, astrocytes respond with an increase in intracellular calcium (Araque and Perea, 2004). This signal is important because it can often spread via second messengers through gap junctions into adjacent astrocytes to generate calcium waves (Bennett et al., 2003) and transmit information to sites distant from its origin. Spontaneous (Parri et al., 2001) and evoked (Porter and McCarthy, 1996; Pasti et al., 1997) calcium increases in astrocytes induce the release, through vesicular and non-vesicular pathways, of active substances termed gliotransmitters (Volterra and Meldolesi, 2005). Gliotransmitters include ATP, glutamate, taurine, and d-serine (Panatier and Oliet, 2006). Once released into the extracellular space, these molecules act on purinergic, glutamatergic, and glycinergic receptors located on adjacent neuronal elements, thereby regulating synaptic function and overall neuronal excitability.

Astrocytes are extensively coupled by gap junctions. A gap junction is an intercellular channel, composed of connexon protein subunits, which acts as a small pore connecting the cytoplasm of two cells (Dermietzel and Spray, 1993). Gap junctions allow passage of small ions between the cells they connect, resulting in electrical coupling. They also allow passage of small molecules up to 1 kDa, including such

signaling molecules as calcium, cAMP, and IP_3. Astrocytic gap junctions are primarily composed of Cx43 (Dermietzel et al., 1991), while the connexins composing gap junctions in neurons may include Cx26 and Cx32 (Dermietzal et al., 1989). Though gap junction communication plays a major role in cellular homeostasis, differentiation, and growth, it is not an exclusive cell-to-cell process. Recent work suggests that unapposed hemichannels may permit the passage of molecules between the cytoplasm and extracellular surroundings (Ebihara, 2003), and that a member of the gap junction protein family, the pannexin channel, might play a specific role in astrocyte biology. Pannexins are implicated in the regulation of astrocytic apoptosis and ATP signaling via the particular purinergic receptor, $P2X_7$ (Locovei et al., 2007).

Hypothalamic astrocytes also communicate via gliotransmitter release. Astrocytes express calcium excitability, which stimulates them to release chemical transmitters such as glutamate and ATP. Just as neurons express excitability as a function of voltage-gated ion channels opening in the plasma membrane, astrocytes express their own form of excitability through elaborate calcium signals and the occurrence of calcium waves. These calcium waves can be restricted to one cell (intracellular) or transmitted to neighboring cells (intercellular) (Scemes and Giaume, 2006). The basic steps that lead to intracellular calcium waves in astrocytes usually involve the activation of G-protein-coupled receptors, activation of phospholipase C, and the production of IP_3, which following IP_3R activation, leads to calcium release from the endoplasmic reticulum (ER). Once triggered, intracellular calcium waves can be transmitted to neighboring cells as intercellular calcium waves.

Purinergic Signaling

Separate families of purinergic receptors for adenosine (P1 receptors) and for ATP and ADP (P2 receptors) exist (Burnstock et al., 1978). P2 receptors belong to two main families: a P2X family of ligand-gated ion channel receptors, and a P2Y family of G-protein-coupled receptors (Abbrachio and Burnstock, 1994). At present, seven P2X and eight P2Y receptor subtypes are recognized, including receptors that are sensitive to pyrimidines as well as to purines, and sugar nucleotides such as UDP-glucose and UDP-galactose (North, 2002; Abbrachio et al., 2003). Members of the family of ionotropic $P2X_{1-7}$ receptors have intracellular N and C termini that have consensus-binding motifs for protein kinases; two transmembrane (TM)-spanning domains, the first (TM1) being involved with channel gating and the second (TM2) lining the ion pore; and an ATP-binding site, which might involve regions of the extracellular loop adjacent to TM1 and TM2 (Fields and Burnstock, 2006). The metabotropic P2Y receptor subtypes ($P2Y_{1,2,4,6,11-14}$) have an extracellular N terminus and an intracellular carboxyl terminus. These receptors have 7 TM-spanning regions, which help to form the ligand-docking pocket. The intracellular loops and C terminus have structural diversity among P2Y subtypes, influencing the degree of coupling with $G_{Q/11}$, G_s, and G_i proteins (Abbrachio et al., 2006). Under certain conditions, P2Y receptors might form homo- and heteromultimeric assemblies, and many tissues express several P2Y subtypes. In response to nucleotide activation, recombinant P2Y receptors either activate PLC and release intracellular calcium, or affect adenylyl cyclase and alter cAMP levels (Abbracchio et al., 2006).

Astrocytes *in situ* and *in vitro* express, at different levels, several ionotropic and metabotropic P2 purinergic receptors, some of which have been implicated in the transmission of calcium signals (Fumagalli et al., 2003). Among the metabotropic P2Y receptors, the P2Y1R and P2Y2R subtypes are likely those predominantly expressed in astrocytes (Ho et al., 1995). Although both of these G-coupled P2Rs generate PLC and IP_3 upon stimulation and thus contribute to generating calcium transients, they differ with regard to their sensitivity and selectivity for nucleotides, with the exception of ATP that is an agonist (in the micromolar range) for both receptors, the purine diphosphate nucleotide ADP is a potent agonist (in the nanomolar range) at the P2Y1R while the pyrimidine triphosphate nucleotide UTP stimulates (in the micromolar range) P2Y2R but not P2Y1R (Burnstock and Knight, 2004).

While the central nervous system contains multiple receptors for nucleotides such as ATP, UTP, ADP, and UDP, the presence of enzymes at the cell surface to readily hydrolyze ATP to form ADP and eventually adenosine is an important process (Wink et al., 2006). Extracellular adenosine acts as a local modulator with a generally cytoprotective function in the body (Fredholm et al., 2001) and is a promising candidate for a sleep-inducing factor: its concentration is higher during wakefulness than during sleep, it accumulates in the brain during prolonged wakefulness, and local perfusions as well as systemic administration of adenosine and its agonists induce sleep and decrease wakefulness (Porkka-Heiskanen, 1999). There are four known subtypes of adenosine receptors (ARs)—referred to as A_1, A_{2A}, A_{2B}, and A_3—each of which has a unique pharmacological profile, tissue distribution, and effector coupling (Jacobson and Gao,

2006). All four subtypes are members of the superfamily of G-protein-couple receptors, and are most closely related to the receptors for the biogenic amines. Extracellular adenosine levels are quite variable, depending on the tissue and the degree of stress experienced, and therefore the basal levels of stimulation of the four subtypes by the endogenous agonist vary enormously. The sources of adenosine include release through an equilibrative transporter, a result of cell damage, or ectonucleotidase-mediated hydrolysis of extracellular adenine nucleotides (Zimmerman, 2000), which have their own signaling properties that are mediated by purinergic P2 receptors. Ectonucleotidases are present on the extracellular surface of many tissues and are crucially involved in numerous important functions (Zimmerman, 2000). For example, in the brain, ectonucleotidases rapidly and effectively shift signaling by released adenine nucleotides and their products to signaling through ARs. Astrocyte-derived adenosine, acting on A_1ARs, has a central role in the integration of synaptic activity by astrocytes that leads to widespread coordination of synaptic networks (Halassa et al., 2009; Pascual et al., 2005). Overall, it is important to understand the relationship between adenosine and ATP signaling in the SCN and their roles in sleep and wakefulness.

ATP Release Mechanisms

Though it is understood that cellular communication is facilitated by ATP release and binding to purinergic receptors, there is debate about the release mechanisms involved. There is evidence for multiple pathways for ATP signaling in glial cells, and this research is designed, in part, to address this idea. Synaptic vesicle release proteins

have been detected in astrocytes, and blocking their function using genetic or pharmacological methods inhibits ATP release, thereby affecting aspects of neuronal physiology (Pascual et al., 2005). Transfection of a glial cell line with the gap junction proteins connexin 43, 32, or 26 increases ATP release and intercellular calcium wave propagation (Cotrina et al., 1998), suggesting that the gap junction proteins that are unpaired with those in adjacent cells (hemichannels) could mediate the release of ATP. However, altering expression of connexins can alter P2Y purinergic receptor expression in astrocytes (Suadicani et al., 2003), which could also affect calcium wave propagation, and many gap junction channel blockers are antagonists of the $P2X_7$ receptor (Suadicani et al., 2006). Other evidence supports a mechanism of ATP release from astrocytes through membrane channels with a large diameter pore, such as the $P2X_7$ receptor, contributing to intercellular calcium waves (Arcuino et al., 2002). ATP is also released from astrocytes during cell swelling (Darby et al., 2003), implicating membrane channels that are involved in osmoregulation or activated by membrane stretch. Altogether, many mechanisms for the release of ATP from cells have been identified in recent years, including vesicular (Bowser and Khakh, 2007) and non-vesicular release mechanisms of ATP (Queiroz et al., 1999).

A central problem in this field of neural cell signaling research is to isolate specific gliotransmitter release mechanisms that operate in a given glial signaling context. In my research, the context is the rhythmically controlled signaling of ATP from SCN hypothalamic cell cultures. Recently, it has been demonstrated that global production and extracellular levels of ATP oscillate in a circadian fashion in SCN2.2 cell

cultures (Womac et al., 2009). The average period (tau) of SCN2.2 oscillations in extracellular ATP levels was 23.0 hours as determined by luciferin-luciferase chemiluminescence detection. Surprisingly, oscillations in this circadian clock-controlled extracellular ATP accumulation were also consistently observed in cortical astrocyte cultures and hepatocyte cell lines. These results suggested that circadian oscillations in extracellular ATP represent a physiological output of the mammalian cellular clock, common to both central pacemakers and peripheral oscillators. Another recent study indicates that rhythmic extracellular ATP in the SCN may be mechanistically linked to the cellular events underlying calcium wave propagation (Cox, 2007). Intercellular calcium waves occur widely among different cell types. A characteristic property of astrocytes grown in cell culture is that a calcium rise in one cell leads to a calcium rise in neighboring cells, thus generating a multi-cellular calcium wave. Activation of metabotropic receptors (e.g. purinergic receptors) in the astrocyte plasma membrane causes a G-protein-dependent activation of phospholipase C, which leads to generation of IP_3. This is turn triggers calcium release from the endoplasmic reticulum (ER), which underlies propagating intercellular and intracellular calcium waves (Volterra et al., 2002). ATP is the primary active messenger in these stimulus-evoked calcium waves (Guthrie et al., 1999). Extracellular ATP accumulation was correlated with significant rhythms in gliotransmission (calcium waves) in SCN2.2 cell cultures, where calcium waves were smaller when endogenous levels of extracellular ATP were higher and vice versa (Cox, 2007). However, exogenously applied ATP reduced the spread of calcium waves at both the endogenous trough and peak in

extracellular ATP accumulation. Therefore, exogenous ATP reduced the spread of calcium waves regardless of the time of application during the endogenous 24 hour ATP accumulation cycle, suggesting an underlying rhythm in purinergic responsiveness of the SCN2.2 cells to the exogenous ATP.

Many questions are raised by the above studies examining clock-controlled ATP signaling and stimulus-evoked ATP-dependent calcium waves. In the following chapters, I will present data that the circadian accumulation of ATP is mediated by calcium signaling mechanisms and not by cell cycle or cell death mechanisms among SCN2.2 cells. Also, by the fact that astrocytes express multiple purinergic receptors with multiple potential cellular signaling actions, I propose that stimulation-evoked ATP signaling and clock-controlled ATP signaling represent distinct neural cell signaling processes in the SCN2.2 cell line and likely the SCN of the mammalian brain.

This study utilizes SCN2.2 cells to investigate the relationship between ATP signaling and calcium signaling and whether these processes are mechanistically linked in the context of ATP accumulation rhythms. First, I hypothesized that multiple ATP release mechanisms exist in SCN2.2 cells. To test this hypothesis, luciferin-luciferase assays and calcium wave studies were conducted while experimentally manipulating cAMP and ATP signaling pathways. Second, I postulated that calcium signaling influences the clock-controlled extracellular ATP accumulation rhythm. To test this hypothesis, I conducted studies using modulators of cytoplasmic, mitochondrial, and endoplasmic reticulum calcium stores to assess the ability of calcium signaling to affect clock-controlled ATP signaling. Finally, I hypothesized that cell cycle and cell death

mechanisms are not responsible in mediating rhythmic ATP accumulation. Therefore, I tested whether these two processes represent regulated physiological mechanisms in phase with the biological clock-controlled accumulation of ATP. Using a cell cycle inhibitor as well as a caspase assay, cell cycle and cell death mechanisms were assessed in relation to the clock-controlled ATP accumulation rhythm.

CHAPTER II

MATERIALS AND METHODS

SCN2.2 Cell Culture General Protocol

SCN2.2 cell cultures were derived from fetal progenitors of the rat SCN (embryonic day 15) immortalized with the adenovirus E1A gene (Earnest et al., 1999). Cells derived from a single passage were expanded onto multiple dishes (60mm; Corning, Corning NY)) coated with mouse laminin (Sigma, St. Louis, MO) and maintained in minimum essential medium (MEM; Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Hyclone, Waltham, MA), glucose (3000 µg/ml), L-glutamine (292 mg/ml), and 1% PSN antibiotic (penicillin, streptomycin, neomycin; Gibco/Invitrogen, Grand Island, NY) under constant temperature (37ºC) and 5% CO_2 conditions. During cell passages, the medium was changed every 48 hours, and the cultures were passaged approximately every two days after they had reached confluent cell density.

Luciferin-Luciferase (Luc/Luc) ATP Assay

Imaging Luc/Luc chemiluminescence with CCD camera. To analyze ATP levels in living cultures, chemiluminescent imaging was performed on SCN2.2 cells that were maintained in an incubator at 37ºC and 5% CO_2 equipped with a liquid nitrogen-cooled CCD camera (Versarray, Photometrics). The CCD was cooled to approximately -110ºC and images were captured using 5 min exposures in total darkness. Intensities of

luminescence from the collected images were analyzed using MetaMorph 4.6 imaging software (Universal Imaging Corporation, Downingtown, PA). Cell-free, chemiluminescence assays of extracellular ATP levels were performed by incubating aliquots (100 µl) of media samples with 1 µl of luciferase and 2 µl of luciferin (Sigma, St. Louis, MO) in wells of a black, 96-well plate (Thermo, Milford, MA).

Quantification of ATP levels in culture media samples with TopCount Luminometer. The TopCount luminometer-based assay was used for studies involving clock-controlled ATP accumulation of SCN2.2 cell cultures, as measured in media samples collected at specific time points. SCN2.2 cell cultures in 60mm plastic dishes were placed in 10% FBS medium. After 24 hours, cells were washed with 5% FBS medium and placed in 5% FBS medium for 24 hours. After 24 hours in 5% FBS medium, the SCN2.2 cell cultures were washed with serum-free neurobasal medium two times and then placed in neurobasal medium for the duration of the study. Timepoint 0 was established after SCN2.2 cells were washed and placed in neurobasal media. Medium was then collected every 2 or 4 hours (dependent on the experiment), placed in a 2 ml cryotube, and snap-frozen in liquid nitrogen. All cryotubes were then placed in a -80 ºC freezer until all samples were collected. Once the time course was complete, the Luc/Luc assay of 1 µl of luciferase and 2 µl of luciferin was added to 100 µl of thawed sample medium already placed in a 96 well black plate. Luc/Luc-loaded samples were read by a TopCount luminometer to quantify ATP chemiluminescence. To quantify ATP from SCN2.2 cultures on 2-well glass chamberslides, cells were placed in 10% FBS medium in 60mm plastic dishes until confluence was obtained. After 24 hours,

cells were trypsinized and plated to 2-well glass chamberslides in 5% FBS medium. Following this event, the time course was started (T0). At T24, the 5% FBS medium was washed out with 2 neurobasal washes and replaced with neurobasal medium. Medium was then sampled every two hours for ATP analysis until ~T52, or until cell density forced termination due to reduction in cell adhesion. Once the time course was complete, the Luc/Luc assay was performed on the samples in the same manner described above.

Generation of ATP standard curve with CCD camera and TopCount Luminometer. To approximate ATP levels in living SCN2.2 cultures and in conditioned culture medium, standard curves were generated for both the CCD-based imaging assay and the TopCount (TC)-based photomultiplier assay using known concentrations of ATP. Chemiluminescence derived from culture media samples was calibrated relative to assay standards ranging from 1 pM to 100 nM ATP in unconditioned medium. Internal controls consisting of unconditioned medium without ATP standard, luciferase, or luciferin were included on all analyzed plates. In this analysis, ATP standards containing FBS exhibited a dose-dependent suppression of chemiluminescent signal and media samples from SCN2.2 cultures containing 10% FBS consistently produced lower signal intensities than those obtained from cultures maintained in serum-free medium.

Luc/Luc assays were also performed to confirm that chemiluminescent activity was dependent on ATP in the culture medium. Treatment with apyrase (50U/ml), an enzyme that degrades ATP, abolished detectable chemiluminescence in the medium

from SCN2.2 cultures, demonstrating that ATP is necessary to drive the Luc/Luc reaction in this assay.

Fluorescence Microscopy Techniques

Ratiometric imaging of stimulus-evoked calcium waves. Confluent astrocyte cultures were sub-cultured onto 2-well glass chamberslides and allowed to grow to confluence (2-3 days). Cells were loaded with 8 μM FURA-2 AM (Molecular Probes, Eugene, OR) in neurobasal medium for 1 hour at 37° C in a 5% CO_2 cell culture incubator. Cells were then washed 6-7 times with neurobasal medium. Calcium imaging was conducted at room temperature with an Olympus IX70 inverted microscope. Images were acquired using a CoolSnapHQ2 camera (Actimetrics, Wilmette, IL) and an entire field of cells was designated as a region of interest (ROI) for analysis. A single glial cell was stimulated using mechanical stimulation with a micromanipulated, glass micropipette to elicit a calcium wave. The area of the spread of the wave was calculated using SimplePCI 6.0 imaging software (Compix, Inc., Cranberry Township, PA). Images were collected approximately every 0.5 seconds for 1 minute in order to permit recording of the initiation and maximal extent of the spread of the calcium wave through each ROI. Even using the 20× objective, the wave occasionally spread beyond the field of view, indicating that the calculated areas of cells involved in the wave most likely underestimated the full extent of wave propagation.

Imaging of cytoplasmic and mitochondrial calcium. To characterize cytoplasmic and mitochondrial calcium levels at given time points, cells were loaded

with either 4 μM FLUO-4 AM (Molecular Probes, Eugene, OR) or 8 μM RHOD-2 AM (Molecular Probes, Eugene, OR) in neurobasal medium for 1 hour at 37° C in a 5% CO_2 cell culture incubator. Cells were then washed 6-7 times with neurobasal medium. Calcium imaging was conducted at room temperature with an Olympus IX70 inverted microscope. Both cytoplasmic (FLUO-4 AM) and mitochondrial (RHOD-2 AM) calcium fluorescence intensities were quantified using SimplePCI 6.0 imaging software (Compix, Inc., Cranberry Township, PA).

Quantifying cell count and caspase activity. To quantify overall cell counts at specific ATP time points, 300 nM 4',6-diamidino-2-phenylindole (DAPI), a fluorescent marker of cell nuclei (Sigma, St. Louis, MO), was loaded for 5 minutes in neurobasal medium at 37° C in a 5% CO_2 cell culture incubator. Cells were then washed 6-7 times with neurobasal medium. DAPI imaging was conducted at room temperature with an Olympus IX70 inverted microscope. The cell counting analysis involved dividing up the collected image into 9 equal regions of interest and numbering those regions. Three numbers were selected randomly and these three regions were then counted. To approximate total number of cells per image, this number was then multiplied by three to account for all nine regions. To quantify caspase activity, a CaspaTag Pan-Caspase In Situ assay kit (Millipore, Billerica, MA) was used to mark active caspase 7. The methodology is based on Fluorochrome Inhibitors of Caspases (FLICA). The inhibitors are cell permeable and non-cytotoxic. Once inside the cell, the inhibitor binds covalently to the active caspase 7. This kit uses a carboxyfluorescein-labeled fluoromethyl ketone peptide inhibitor of caspase (FAM-LEHD-FMK), which produces a green fluorescence.

When added to a population of cells, the FAM-LEHD-FMK probe enters each cell and covalently binds to a reactive cysteine residue that resides on the large subunit of the active caspase heterodimer, thereby inhibiting further enzymatic activity. The bound labeled reagent is retained within the cell, while any unbound reagent will diffuse out of the cell and is washed away. The green fluorescent signal is a direct measure of the amount of active caspase present in the cell at the time the reagent was added. Following FLICA incubation, DAPI was added for 5 minutes to each cell culture. Imaging was conducted with an Olympus IX70 inverted microscope and images were captured and analyzed using SimplePCI software. Cells were counted as described above.

Characterization of Clock-Controlled and Stimulus-Evoked ATP Accumulation

Prior to experimental analysis, SCN2.2 cells were propagated as described above. To allow quantification of ATP by Luc/Luc assay, SCN2.2 cells were put through a serum reduction protocol. The serum reduction involved transferring living cultures of SCN2.2 cells from medium containing 10% FBS after 48 hours to medium containing 5% FBS for 24 hours followed by transfer to serum-free neurobasal medium with B-27 supplements. SCN2.2 cells were grown to confluence on 60mm dishes while undergoing the serum reduction protocol described above. Then, luciferin and luciferase were added to the neurobasal medium. To evoke stimulation of ATP accumulation, 30-50 µM diameter glass micro beads (Polysciences Inc., Warrington, PA) were dropped onto the confluent cells and subsequent production of light via the luciferin/luciferase

assay was captured by a cryo-cooled liquid nitrogen CCD camera. Clock-controlled ATP accumulation in SCN2.2 cultures was imaged with the CCD camera as the basal (unstimulated) production of ATP chemiluminescence over time.

Analysis of cAMP Signaling in the Regulation of ATP Accumulation

In both stimulus-evoked and clock-controlled ATP accumulation studies, 15 μM of forskolin (FSK) (Sigma, St. Louis, MO) in 0.1% DMSO (Sigma, St. Louis, MO) was added to SCN2.2 cell cultures to activate adenylate cyclase and the production of cAMP. To perform calcium wave studies, our physiological measure of stimulus-evoked ATP release, confluent SCN2.2 cells were incubated with 8 μM of FURA-2 AM, a fluorescent calcium indicator, for one hour prior to washout. Parallel SCN2.2 cultures on glass coverslip chamber dishes were treated with 0.1% DMSO (control) for one hour at 37°C with 5% CO_2. Cells were washed with neurobasal media and fresh neurobasal media was added to each well prior to imaging.

In stimulation-evoked ATP accumulation/calcium wave studies, cells were placed under an inverted Olympus IX70 microscope, manually stimulated with a micromanipulator-controlled glass micropipette tip to focally elicit a calcium wave, and ratiometric calcium imaging was conducted. Data analysis was performed using SimplePCI 6.0 software, which captured each calcium wave as a sequence of images over a 30 second time period. The software allowed for analysis of wave properties such as maximum area of spread and rate of spread to be measured and analyzed.

In clock-controlled ATP accumulation studies, rather than examining calcium wave spread, the luciferin-luciferase ATP assay was conducted on separate unstimulated (i.e., not evoked with external cues) SCN2.2 cells treated with FSK and DMSO in 24 well plastic dishes. A liquid nitrogen cryo-cooled CCD camera was used to capture and quantify the basal production of ATP-dependent chemiluminescence.

Determination of Purinergic Clock-Controlled Signaling Pathways in ATP Accumulation

In both stimulus-evoked and clock-controlled ATP signaling studies, purinergic receptor antagonists were added to the culture medium to examine their effect on ATP-dependent calcium waves (stimulus-evoked) and basal levels of ATP accumulation (clock-controlled). DMSO (control), suramin (100 μM, Sigma, St. Louis, MO), a general P2 purinergic receptor antagonist, PPADS (100 μM, Tocris, Ellisville, MO), a selective P2Y receptor antagonist, and BBG (100 nM, Sigma, St. Louis, MO), a selective $P2X_7$ receptor antagonist, were added to the medium for 1 hour in 24 well plastic dishes before luciferin-luciferase chemiluminescence images were captured with a CCD camera. In evoked ATP accumulation studies, SCN2.2 cells were cultured in glass coverslip chamber slides and treated separately with these purinergic antagonists. Following incubation with FURA-2 AM, stimulus-evoked calcium waves were elicited with a glass micropipette and ratiometric calcium imaging was performed.

Determination of Intracellular Calcium Signaling During Clock-Controlled ATP Accumulation

SCN2.2 cells were plated and ATP accumulation was analyzed via Luc/Luc assay as described above. Resting calcium levels were quantified with FURA-2 AM, a fluorescent calcium indicator. Neurobasal medium samples were collected from each glass well at time points T40 and T50. FURA-2 AM incubation was 1 hour, followed by multiple washes of neurobasal medium. In addition, 8 μM of BAPTA AM, a calcium-specific chelator (Molecular Probes, Eugene OR), was added to separate cultures at T24. FURA-2 AM incubation for 1 hour followed at T40 and T50 time points, respectively. Calcium wave studies were carried out on BAPTA AM cultures, as described above. Both FURA-2 AM and BAPTA AM cultures were imaged on an inverted Olympus IX70 microscope and analyzed with SimplePCI 6.0. FURA-2 AM intensities were quantified at both T40 and T50 time points. Calcium wave area was quantified for BAPTA AM studies.

Dependence of Clock-Controlled ATP Accumulation on ER Calcium Stores

Cells were plated and analyzed via Luc/Luc assay as described above. Cells experiencing a peak or trough in ATP levels (confirmed by luciferin-luciferase assay) were treated separately with thapsigargin (1 μM; Sigma), an inhibitor of the sarco/endoplasmic reticulum calcium ATPase, or SERCA. Ratiometric calcium imaging with FURA-2 AM was conducted on all cultures and calcium wave area was analyzed as described above.

Dependence of Clock-Controlled ATP Accumulation on Cytoplasmic and Mitochondrial Calcium

Cells were plated and analyzed via Luc/Luc assay as described above. Since ATP is produced in mitochondria and calcium signaling is important to mitochondrial function, we quantified mitochondrial calcium levels with RHOD-2 AM, a fluorescent indicator of intramitochondrial calcium. We also quantified cytoplasmic calcium levels with FLUO-4 AM, a fluorescent indicator of cytoplasmic calcium. At certain time points T40 and T50, medium samples were collected from each glass well containing cells before FLU0-4 AM and RHOD-2 AM incubation for 1 hour. After 1 hour, glass wells were washed out with neurobasal medium multiple times before imaging. In addition, Ru360 (2 μM; Sigma), a specific mitochondrial calcium uptake inhibitor, was added to separate SCN2.2 cell cultures at T24. Since Ru360 does not affect other cellular calcium transport processes, the correlation of mitochondrial calcium signaling with the clock-controlled ATP accumulation rhythm was directly tested. Following medium sampling at T40 and T50 from separate cultures, Ru360-treated SCN2.2 cells were incubated with RHOD-2 AM for 1 hour. FLUO-4 AM, RHOD-2 AM, and Ru360 cultures were imaged on an inverted Olympus IX70 microscope and analyzed with SimplePCI 6.0. Fluorescent intensities of FLUO-4 AM, RHOD-2 AM, and Ru360-treated cultures loaded with RHOD-2 AM were quantified at both T40 and T50 time points.

Dependence of Clock-Controlled ATP Accumulation on Cell Cycle

SCN2.2 cell cultures were grown on plastic 60mm dishes per the sampling protocol described above. Medium samples were taken every four hours for a total of 72 hours for the luciferin-luciferase assay to determine extracellular ATP accumulation. At T24, the cell cycle inhibitor arabinosylcytosine (AraC), an anti-cancer drug that prevents cell division (100 μM; Sigma), was added to select cell cultures. After addition of AraC, medium sampling took place over the next 24 hours. AraC was then added again at the 48 hour time point. Another 24 hours of media sampling commenced to take the cells out a full 72 hours. To test whether AraC was indeed inhibiting the SCN2.2 cells from dividing, SCN2.2 cell cultures were plated on glass 2 well chamberslides per the imaging protocol described above. AraC was then added at T24 and medium samples were taken from separate cultures at T40 and T50, respectively. Following medium sampling, cell cultures were incubated in DAPI for 5 minutes to mark all cell nuclei as well as cell nuclei undergoing mitotic cell division. After incubation in DAPI, cells were washed with neurobasal medium and then examined with an inverted Olympus IX70 fluorescence microscope for image capture. The total number of cell nuclei for each treatment group was counted and compared at T40 and T50 time points, respectively.

Dependence of Clock-Controlled ATP Accumulation on Rhythmic Cell Death

Cells were plated and analyzed via Luc/Luc assay as described above. A CaspaTag Pan-Caspase Assay (Millipore) was used to mark active caspase 7 in the cell cultures. In separate cultures at T40 and T50, medium sample was taken and FLICA incubation occurred for 1 hour. After 4-5 washes with neurobasal medium, SCN2.2 cultures were then incubated in DAPI for 5 minutes. Following DAPI incubation, cells were again washed 4-5 times with neurobasal medium and then imaged with an inverted Olympus IX70 microscope. For each timepoint, the total number of DAPI-positive cells were counted to generate a total cell count. Also, the total number of caspase (FLICA) positive cells were counted. To determine caspase activity at a certain timepoint, the total number of caspase-positive cells was divided into the total number of DAPI-positive cells to establish a ratio of apoptotic activity at that timepoint. Apoptotic activity was compared at the T40 and T50 time points, respectively.

CHAPTER III

RESULTS

Characterization of Clock-Controlled and Stimulus-Evoked ATP Accumulation

SCN2.2 cell cultures were bathed in a Luciferin/Luciferase (Luc/Luc) solution and imaged under a cryo-cooled liquid nitrogen CCD camera to determine whether stimulation-evoked and basal extracellular ATP accumulation could be distinguished. ATP was quantified in 18 independent SCN2.2 cultures following a complete medium exchange and 30 minute incubation in the Luc/Luc assay solution. ATP-dependent chemiluminescence was detected in all SCN2.2 cell cultures. Although luminescence intensity varied among the independent cultures tested, all intensities were detectably higher than negative control dishes containing cells and no Luc/Luc assay solution or assay solution in medium without cells (Figure 1A). Apyrase, an enzyme that degrades ATP, decreased detectable chemiluminescence when added to the culture medium during the 30 minute ATP accumulation period. Luminescence intensity was reduced by approximately 97% in apyrase-treated cells when compared to SCN2.2/Luc/Luc controls (Fig. 1B), demonstrating that ATP must accumulate extracellularly to drive the luciferin/luciferase reaction in this assay.

Following quantification of unstimulated basal ATP accumulation, SCN2.2 cultures bathed in luciferin and luciferase were stimulated by glass microbeads dropped from a micropipette (Fig. 2A). ATP chemiluminescence in the mechanically stimulated cells was detected above basal levels of ATP-dependent chemiluminescence. ATP

accumulation was quantified by the generation of an ATP luciferin/luciferase standard curve (Fig. 2B). Basal ATP accumulation ranged from 1 pM to 10 nM in unstimulated cultures, similar to an *in vivo* environment. Stimulated (evoked) ATP chemiluminescence exceeded that of basal SCN2.2 cultures, reaching levels of 50-100 nM ATP concentration.

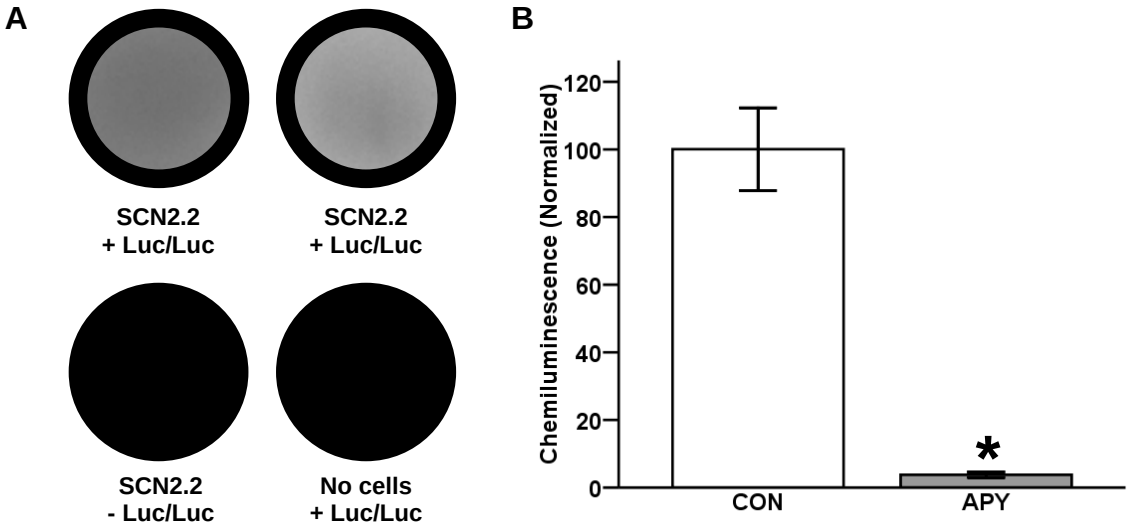

Fig. 1. The presence of ATP in the culture medium was imperative for functionality of the luciferin/luciferase assay. **A)** Representative images of individual wells from a 24 well plate, ATP assay. The top two wells represent unstimulated SCN2.2 cell cultures incubated with luciferin and luciferase to produce a chemiluminescent reaction captured by the CCD camera. The bottom two wells represent negative controls, where SCN2.2 cells were present but no Luc/Luc (*Left*) and where no SCN2.2 cells were present but Luc/Luc was loaded into the medium (*Right*). **B)** The addition of apyrase (APY), an enzyme that degrades ATP, significantly reduced detectable chemiluminescence (*$p<0.05$; $n=4$). Error bars represent 95% confidence intervals.

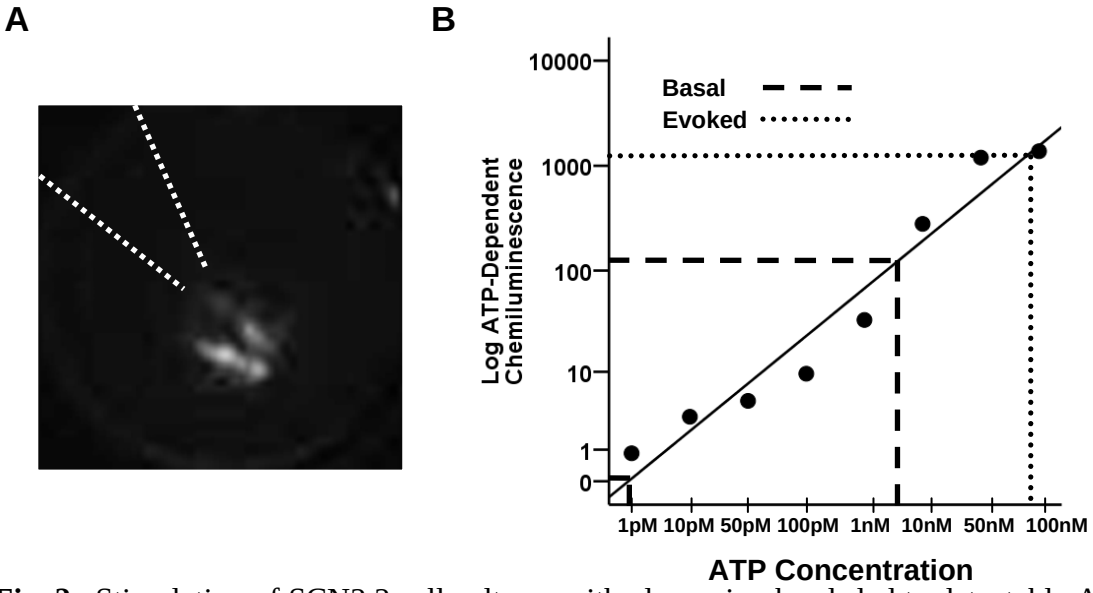

Fig. 2. Stimulation of SCN2.2 cell cultures with glass microbeads led to detectable ATP chemiluminescence. **A)** By dropping glass microbeads onto SCN2.2 cell cultures loaded with luciferin and luciferase, ATP chemiluminescence was detected by a cryo-cooled CCD liquid nitrogen camera. Along with a basal, constitutive ATP signaling mechanism described above, this finding represents a second form of ATP signaling; a stimulus-evoked ATP signaling mechanism following the addition of glass microbeads.
B) ATP luciferin/luciferase assay standard curve of unstimulated and stimulated SCN2.2 cell cultures. Based on comparisons with known concentrations of ATP, unstimulated (basal) ATP levels quantified by cryo-cooled CCD camera image capture ranged from approximately 1 pM up to 10 nM in unstimulated SCN2.2 cell cultures, similar to an *in vivo* environment. Stimulated (evoked) SCN2.2 cell culture chemiluminescence exceeded that of basal cultures, reaching levels of 50-100 nM ATP concentration.

Analysis of cAMP Signaling in the Regulation of ATP Accumulation

cAMP-dependent signaling affects the spread of ATP-dependent calcium waves in astrocytes (Peters et al., 2005). Here, I tested the hypothesis that cAMP signaling also modulates ATP gliotransmission in SCN2.2 cell cultures using forskolin (FSK) to activate adenylate cyclase and elevate cAMP levels. The Luc/Luc assay determined basal accumulation in SCN2.2 cultures treated with FSK and dimethyl sulfoxide

(DMSO) vehicle. ATP chemiluminescence in unstimulated cells (i.e., not mechanically evoked) was decreased by approximately 50% in cells treated with FSK ($p<0.05$; n=18) in comparison to those treated with DMSO vehicle (n=18) (Fig. 3A, B). Therefore, experimental elevation of cAMP leads to a reduction in basal extracellular accumulation of ATP in SCN2.2 cell cultures.

SCN2.2 cell cultures were placed on the stage of an inverted fluorescence microscope and manually stimulated with a glass micropipette to elicit intercellular calcium waves. Calcium waves were monitored using FURA-2 AM ratiometric imaging and the maximum area of calcium wave spread was calculated. Calcium wave propagation of FSK (n=15) and DMSO (n=14) treatment groups was not significantly different ($p>0.05$) (Fig. 3C, D). Therefore, cAMP-dependent signaling modulates basal ATP extracellular accumulation, but not ATP-dependent calcium wave propagation in SCN2.2 cell cultures.

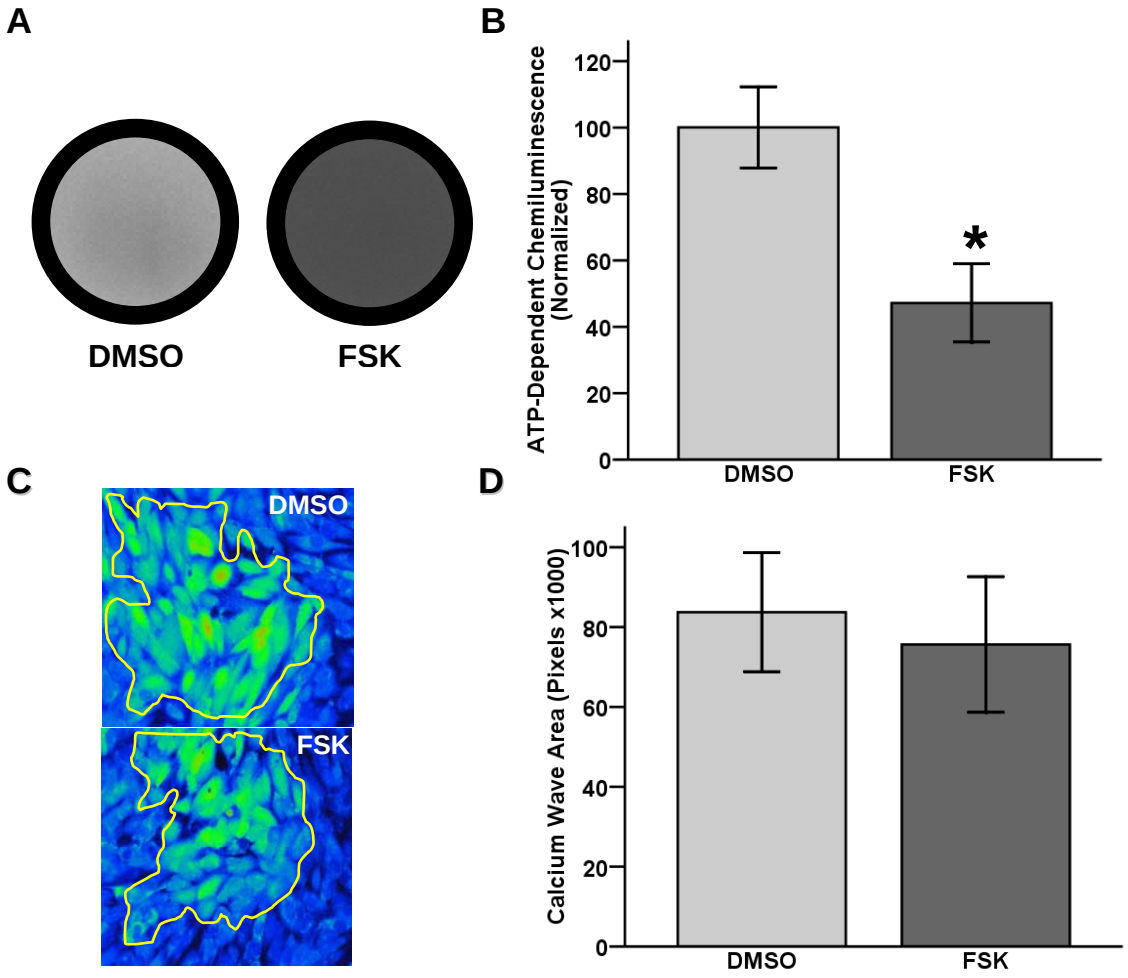

Fig. 3. The upregulation of cAMP signaling modulated basal extracellular ATP accumulation, but not stimulus-evoked ATP signaling among SCN2.2 cells. **A)** Representative images of ATP-dependent chemiluminescence in cell cultures either treated with control vehicle (0.1% DMSO) or 15 µM forskolin (FSK) taken with a cryo-cooled CCD liquid nitrogen camera. **B)** ATP chemiluminescence intensities were significantly reduced in FSK-treated cultures (*$p<0.05$; n=18) in comparison with DMSO-treated SCN2.2 cell cultures (n=18). **C)** Representative pseudocolor images of stimulation-evoked calcium waves in cell cultures loaded with FURA-2 AM and treated with DMSO or FSK. **D)** Calcium wave propagation was not significantly different in FSK-treated cultures ($p>0.05$; n=15) in comparison with DMSO (n=14). Error bars represent 95% confidence intervals.

Determination of Purinergic Signaling Pathways in Clock-Controlled ATP Accumulation

ATP binds purinergic receptors found on the cell membrane to activate intracellular events such as calcium waves. Therefore, I added purinergic receptor antagonists to culture medium to examine their effect on stimulus-evoked calcium wave spread and clock-controlled (basal) extracellular ATP accumulation. Suramin, a general P2 purinergic receptor antagonist, and PPADS, a selective P2Y receptor antagonist, but not BBG, a selective $P2X_7$ receptor antagonist, reduced the spread of calcium waves in SCN2.2 cultures. Calcium wave spread in Suramin-treated (n=10) and PPADS-treated cultures (n=6) was approximately 70% lower than wave spread of control DMSO-treated cells ($p<0.05$; n=14) (Fig. 4). The propagation of calcium waves and the maximal area of waves in BBG-treated cultures was not significantly different than control waves.

In contrast to stimulus-evoked calcium waves, the addition of BBG to the SCN2.2 cell cultures (n=6) resulted in a 2.5 fold increase in basal extracellular ATP accumulation, as indicated by Luc/Luc chemiluminescence, compared to DMSO controls ($p<0.05$; Fig. 5). Also, the addition of Suramin (n=6) to the cultures caused an approximate 90% reduction in extracellular ATP accumulation, a decrease to almost untraceable levels. PPADS treatment (n=6) did not result in a significant change in chemiluminescence from controls culture values. Thus, disparate purinergic receptor-mediated mechanisms are likely involved in the regulation of basal extracellular ATP accumulation and stimulus-evoked ATP release.

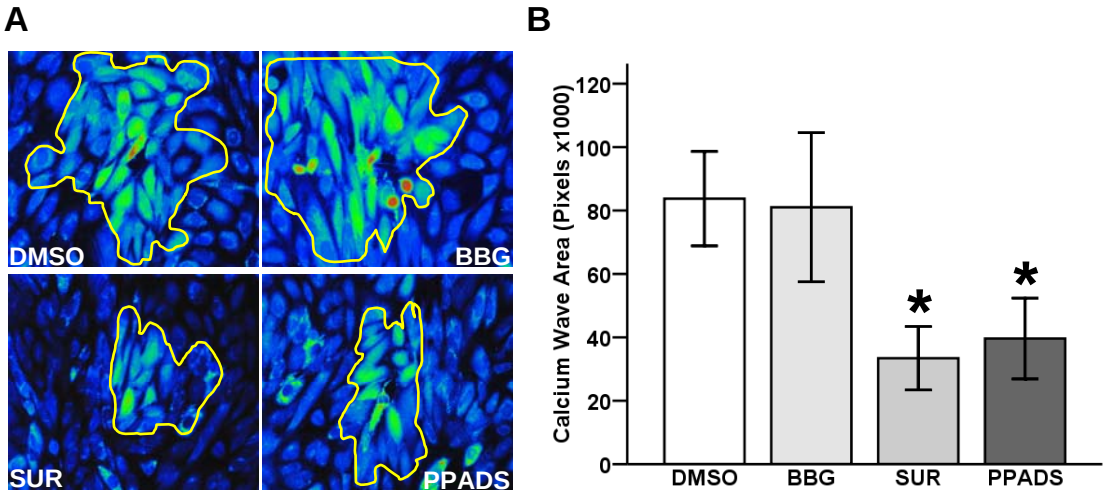

Fig. 4. Stimulus-evoked ATP-dependent calcium wave propagation was dependent on the P2Y purinergic receptor in SCN2.2 cells. **A)** Representative pseudocolor images of stimulation-evoked calcium waves in cell cultures treated with DMSO, BBG, Suramin (SUR), or PPADS. Cells were loaded with 8 µM FURA-2 AM prior to imaging. **B)** Suramin (n=10), a general purinergic receptor antagonist, and PPADS (n=8), a specific P2Y receptor antagonist, significantly reduced calcium wave area in SCN2.2 cell cultures (*$p<0.05$). Error bars represent 95% confidence intervals.

Defining Clock-Controlled ATP Accumulation Peaks and Troughs on 2-Well Glass Chamberslides

A few physiological circadian rhythms have been demonstrated for the master circadian pacemaker cells located within the suprachiasmatic nuclei (SCN) of the mammalian brain. Recent studies have suggested that SCN2.2 cell cultures and cells of the rat SCN *in vivo* may rhythmically release ATP (Womac et al., 2009). These authors demonstrated a rhythm in ATP accumulation in the culture medium of SCN2.2 cells with a period of approximately 24 hours. Furthermore, a rhythm in extracellular ATP accumulation was detected by microdialysis in the rat SCN *in vivo* that persists in

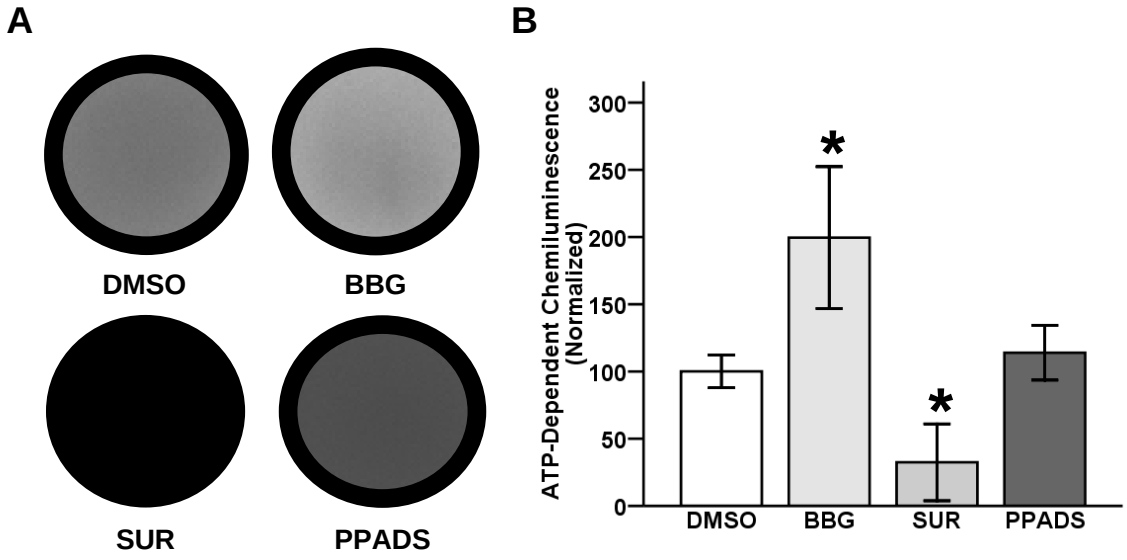

Fig. 5. Basal extracellular ATP accumulation was affected by P2X receptor antagonism, but not P2Y-specific inhibition. **A)** Representative images of SCN2.2 cell cultures in 24 well plates treated with DMSO, BBG, Suramin (SUR), and PPADS. **B)** BBG-treated cultures significantly increased ATP chemiluminescence (*$p<0.05$; n=12) while suramin-treated cultures significantly reduced ATP chemiluminescence (*$p<0.05$; n=15) in comparison to DMSO controls. The ATP intensity of PPADS was not significantly different from DMSO controls. Error bars represent 95% confidence intervals.

constant conditions. Therefore, rhythms in extracellular ATP accumulation are circadian and clock-controlled. In the present study, I have developed a glass coverslip culture system for the coupling of imaging experiments and ATP assay. By defining the rhythmic pattern of ATP accumulation in this approach, specific peaks and troughs in ATP accumulation *in vitro* could be targeted.

Luc/Luc assays on media samples revealed robust and consistent ATP trough and peak time points at approximately 18 to 24h, 30-36h, 40-46h, and 50-56h from the final serum reduction into neurobasal medium (considered time=0) (Fig. 6A). Although the phase relationships of peak and trough ATP levels, as determined from media samples,

were variable from experiment to experiment, they consistently occurred at 12 hour intervals from time zero. Therefore, experimental time points are referred to hereafter as peak ATP and trough ATP, as verified by luminometry. Cell cultures derived from the same passage and handled identically exhibited consistent phase-locked ATP rhythms such that group mean luminescence intensity was significantly greater at peak versus trough time points (Fig. 6B).

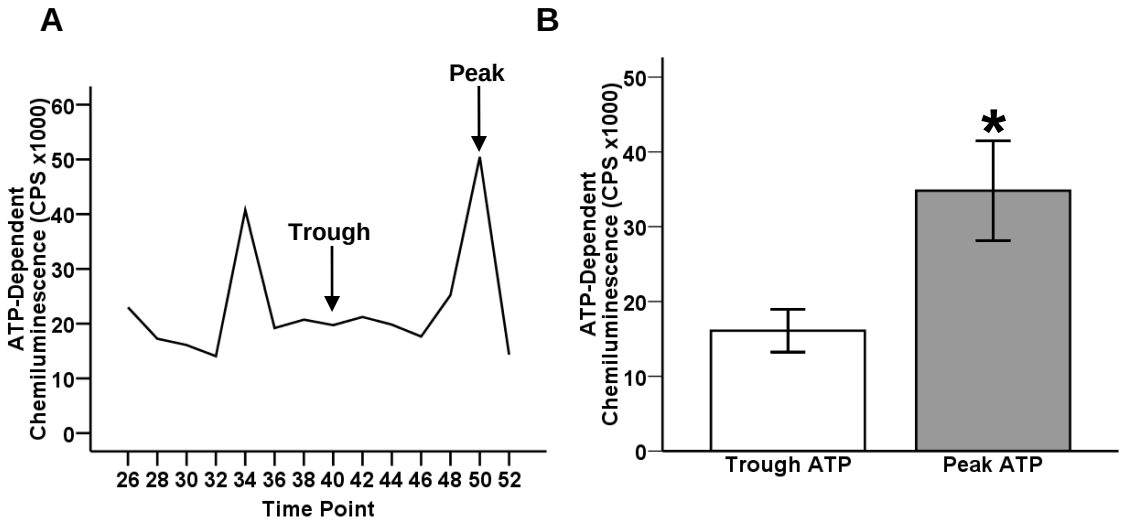

Fig. 6. Extracellular ATP accumulation in glass chamberslide cultures consistently exhibited ATP peaks and troughs with 24 hour periodicity. **A)** Representative ATP chemiluminescence of one replicate SCN2.2 cell culture whose medium was sampled every two hours for luciferin/luciferase assay. **B)** Parallel cultures that were cultured and assayed simultaneously were all in phase as shown by a significant 2-fold increase in ATP-dependent chemiluminescence between the T40 time point and the T50 time point across all cultures (*$p<0.05$; $n=6$). Error bars represent 95% confidence intervals.

Resting Cytoplasmic Calcium Signaling and Clock-Controlled ATP Accumulation

Stimulus-evoked ATP release from astrocytes is vesicular and calcium-mediated (Fumagalli et al., 2003; Pascual et al., 2005). Therefore, I investigated whether calcium signaling plays a role in the circadian-regulated, basal accumulation of extracellular ATP. SCN2.2 cells were grown to confluence on glass coverslip chamberslides, loaded with FURA-2 AM, a cytoplasmic calcium fluorophore, and imaged at both the ATP trough and ATP peak time points, as confirmed by luminometry. Resting cytosolic calcium levels were significantly higher at the ATP trough ($p<0.05$; $n=4$) than at the ATP peak ($n=4$) (Fig. 7), indicating an inverse relationship between extracellular ATP accumulation and resting calcium levels in SCN2.2 cultures.

To determine a direct correlation between extracellular ATP and cytoplasmic calcium levels, BAPTA AM, a calcium specific chelator, was added to the medium of SCN2.2 cell cultures. After cells were loaded with FURA-2 AM, calcium wave imaging was conducted at both the peak and trough ATP time points (Fig. 8). At peak ATP accumulation, the calcium wave spread of the BAPTA-treated cell cultures was approximately 60% reduced compared to waves in control SCN2.2 cell cultures ($p<0.05$; $n=4$). At the trough ATP time point, the calcium wave spread of the BAPTA-treated cell cultures was approximately 50% lower than controls ($p<0.05$; $n=4$). Although calcium wave area was decreased by BAPTA calcium chelation, BAPTA-treated cultures exhibited ATP troughs and ATP peaks in a similar phase relationship to control cultures, although the amplitude of ATP-dependent chemiluminescence was dampened.

Therefore, chelation of cytoplasmic calcium does not dramatically alter rhythmic ATP accumulation.

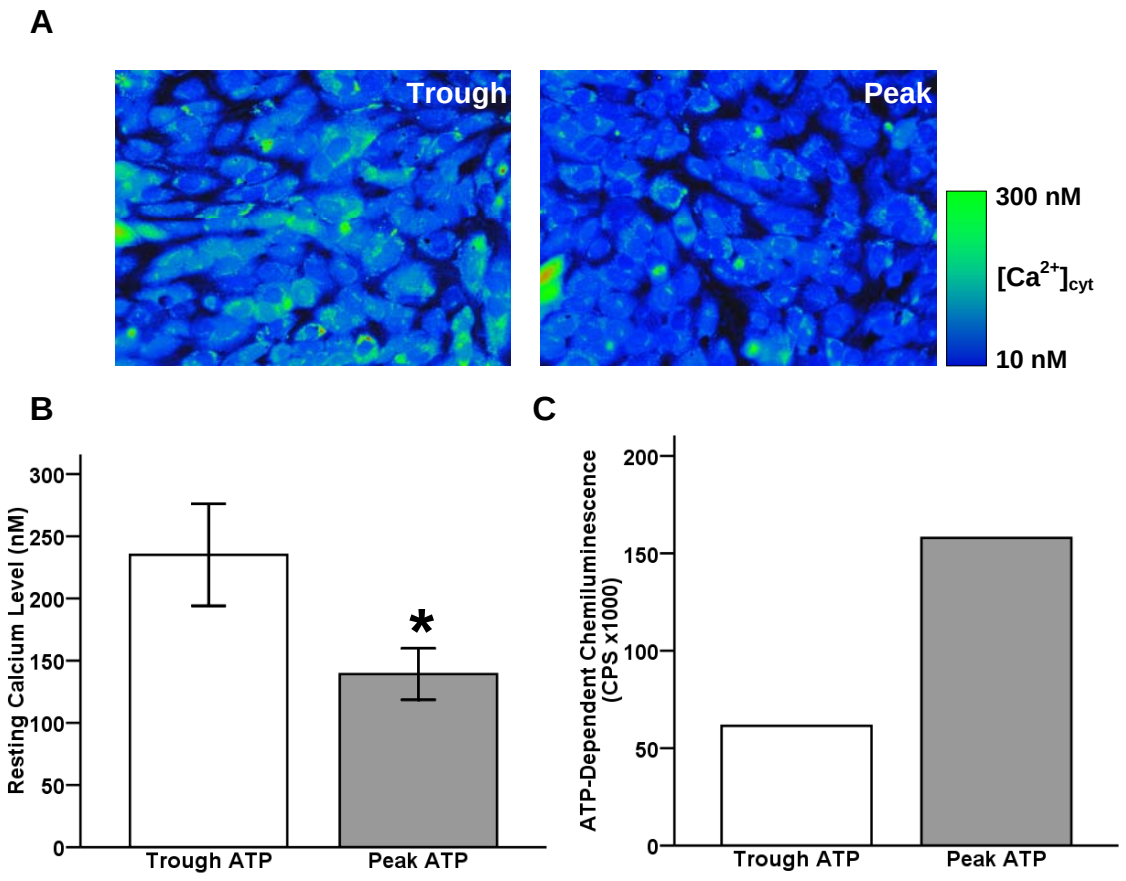

Fig. 7. The resting calcium levels among SCN2.2 cells were significantly different between ATP trough and ATP peak time points. **A)** Representative pseudocolor images of resting cytosolic calcium ($[Ca^{2+}]_{cyt}$) in cultures loaded with 8 µM FURA-2 AM at ATP trough and ATP peak time points. **B)** Resting calcium levels were significantly different at the ATP trough and the ATP peak time point across all cultures (*$p<0.05$; $n=4$). **C)** ATP-dependent chemiluminescent assay revealed a defined ATP trough and ATP peak. Error bars represent 95% confidence intervals.

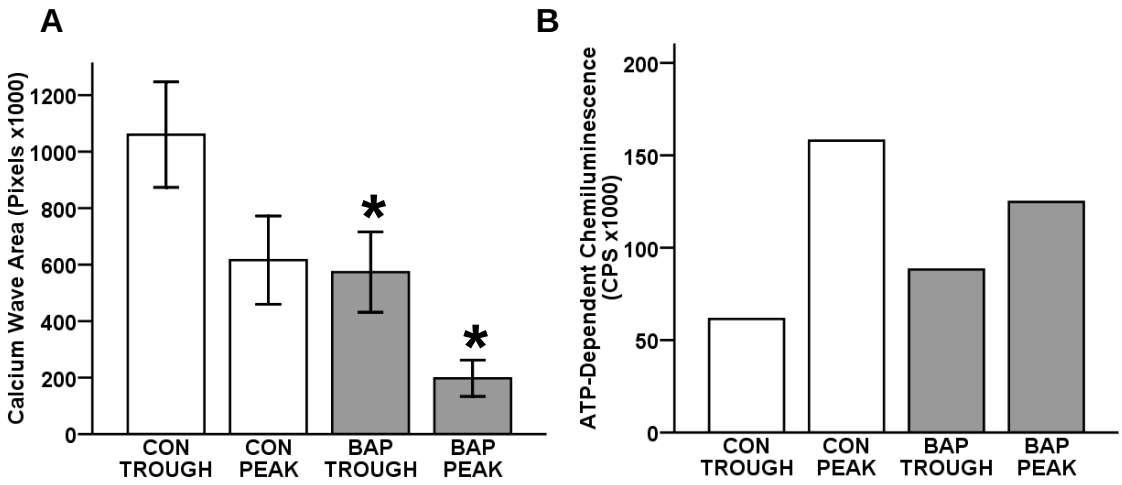

Fig. 8. The addition of BAPTA-AM, a calcium chelator, to SCN2.2 cell cultures led to a decrease in calcium wave area and dampened ATP rhythm. **A)** The area of the calcium waves of the BAPTA-treated SCN2.2 cell cultures was significantly reduced compared with the area of the control calcium waves at both ATP trough and ATP peak time points (*$p<0.05$; n=12). **B)** ATP peak and trough existed for both control and BAPTA-treated SCN2.2 cell cultures (n=4). However, the amplitude of the ATP accumulation rhythm was dampened in the BAPTA-treated cultures. Error bars represent 95% confidence intervals.

Clock-Controlled ATP Accumulation Is Not Dependent on ER Calcium Stores

The endoplasmic reticulum (ER) is a multifaceted organelle that regulates protein synthesis and trafficking, cellular responses to stress, and intracellular calcium levels. Since the ER is important in triggering, propagating, and modulating calcium waves among astrocytes (Charles et al., 1991), I quantified calcium wave area after loading SCN2.2 cell cultures with 1 μM thapsigargin (TG). At the ATP trough, the calcium wave area was significantly decreased in TG-treated cultures ($p<0.05$; n=12) (Fig. 9). It is interesting to note that while calcium wave area was decreased by TG at the ATP

trough time point, TG had no obvious effect on ATP accumulation or the phase relationship of the clock-controlled ATP accumulation rhythm.

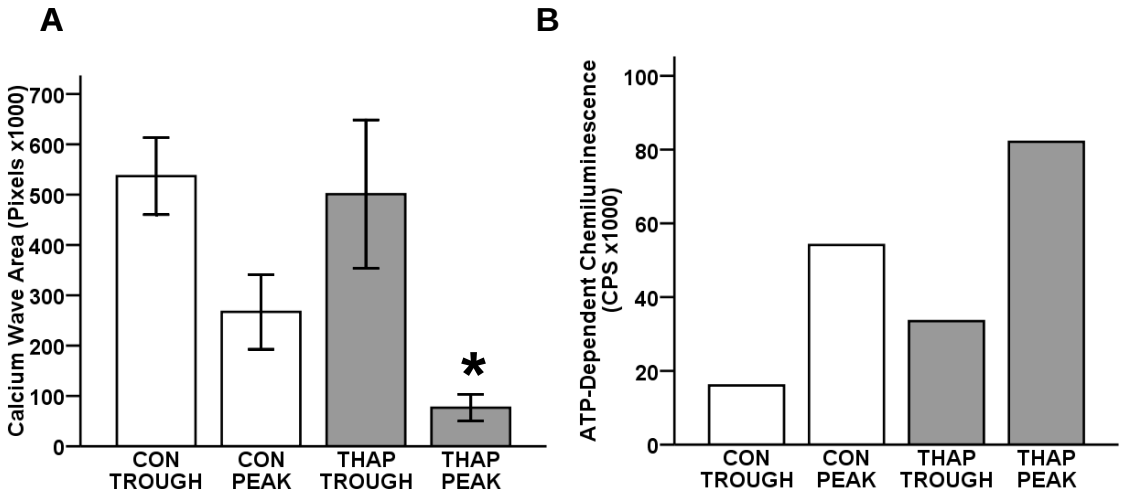

Fig. 9. The addition of thapsigargin, an inhibitor of SERCA pumps, to SCN2.2 cell cultures led to modulation in calcium wave area. **A)** At the ATP peak, the calcium wave area was significantly decreased in thapsigargin-treated cultures compared with controls (*$p<0.05$; $n=12$). **B)** While calcium wave area was decreased by thapsigargin at the ATP peak time point, thapsigargin had no obvious effect on the phase relationship of the clock-controlled ATP rhythm, although the amplitude of extracellular ATP was moderately elevated at both peak and trough time points in thapsigargin-treated cultures. Error bars represent 95% confidence intervals.

Dependence of Clock-Controlled ATP Accumulation on Cytoplasmic and Mitochondrial Calcium

Since ATP is produced in the mitochondria and calcium signaling is important to mitochondrial function, we quantified mitochondrial calcium levels with Rhod-2 AM, a fluorescent indictor of calcium, while monitoring cytoplasmic calcium levels with FLUO-4 AM (Fig. 10). At the ATP trough, as determined by media sample

luminometry, cytoplasmic calcium, consistent with the previous FURA-2 experiment, was significantly reduced at the ATP peak time point ($p<0.05$; $n=6$). However, mitochondrial calcium was increased by approximately 5-fold at the ATP peak time point ($p<0.05$; $n=6$).

Ru360, a specific inhibitor of the mitochondrial calcium uniporter, was added to SCN2.2 cell cultures. Since Ru360 does not affect other cellular calcium transport processes, the correlation of mitochondrial calcium signaling with the clock-controlled ATP accumulation rhythm was directly tested. The addition of Ru360 to SCN2.2 cell cultures resulted in a significant decrease of extracellular ATP chemiluminescence at both trough and peak ATP time points ($p<0.05$; $n=10$) (Fig. 10D). These studies demonstrate that peaks in clock-controlled extracellular ATP accumulation are correlated with elevations in mitochondrial calcium accumulation, whereas troughs in ATP accumulation are coincident with periods of elevated cytosolic calcium levels. Therefore, pharmacological disruption of the mitochondrial calcium uniporter, and thereby ATP production, abolishes rhythmic extracellular ATP accumulation.

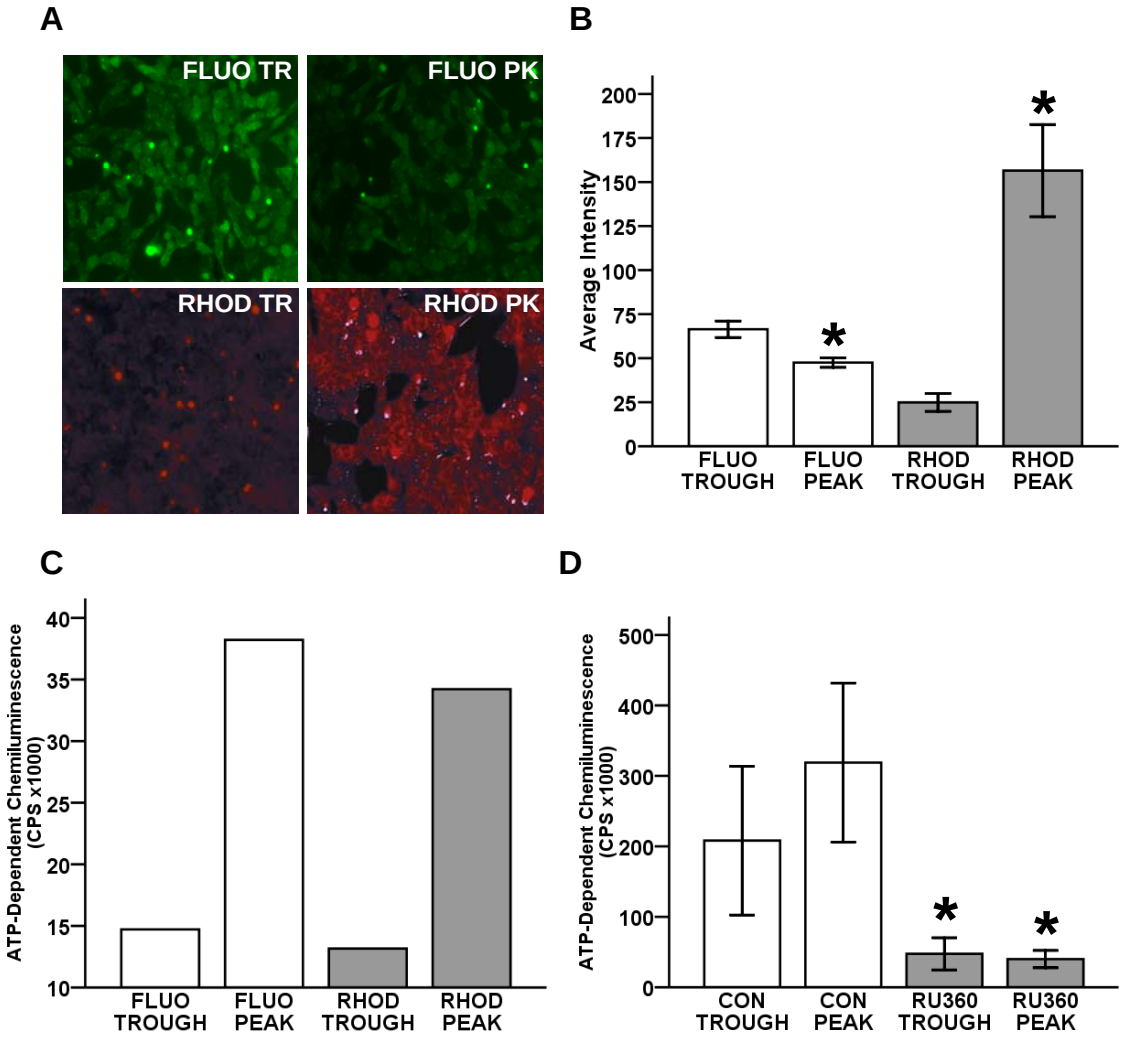

Fig. 10. Cytoplasmic calcium levels were low when extracellular ATP accumulation was high, while mitochondrial calcium levels correlated with extracellular ATP accumulation levels. **A)** Representative images of SCN2.2 cell cultures loaded with Fluo-4 AM, a fluorescent cytoplasmic calcium marker, and RHOD-2 AM, a fluorescent mitochondrial calcium indicator, at the ATP trough (TR) and ATP peak (PK) time points. **B)** At the ATP trough time point cytoplasmic calcium intensities were significantly greater than those at the ATP peak time point (*$p<0.05$; $n=4$). Mitochondrial calcium handling is the opposite of cytoplasmic calcium handling, as mitochondrial calcium levels are significantly reduced at the ATP trough in comparison to mitochondrial calcium levels at the ATP peak (*$p<0.05$; $n=4$). **C)** The luciferin/luciferase assay confirmed that ATP troughs and ATP peaks are present in both media samples of FLUO-4 AM and RHOD-2 AM experimental cultures. **D)** Ru360, a specific mitochondrial calcium uniporter inhibitor, abolished extracellular ATP accumulation and created a loss of the phase relationship between the ATP trough and ATP peak time points (*$p<0.05$; $n=8$). Error bars represent 95% confidence intervals.

Clock-Controlled ATP Accumulation Is Not Dependent on Cell Cycle

ATP (Marcussen and Larsen, 1996), calcium (Baran, 1996), and the biological clock (Gery et al., 2006) can each affect cell division. Consequently, it was important to explore whether antagonizing cell division would abrogate rhythmic ATP accumulation. SCN2.2 cell cultures were grown on plastic 60mm dishes and medium samples were collected every four hours for a total of 72 hours. At 24 hours of culture and thereafter, cultures were treated with the cell cycle inhibitor, AraC. The control (untreated) cultures (n=5) yielded ATP peaks approximately 24 hours apart (Fig. 11). Pharmacologically disrupting the cell cycle with AraC did not affect the extracellular ATP accumulation

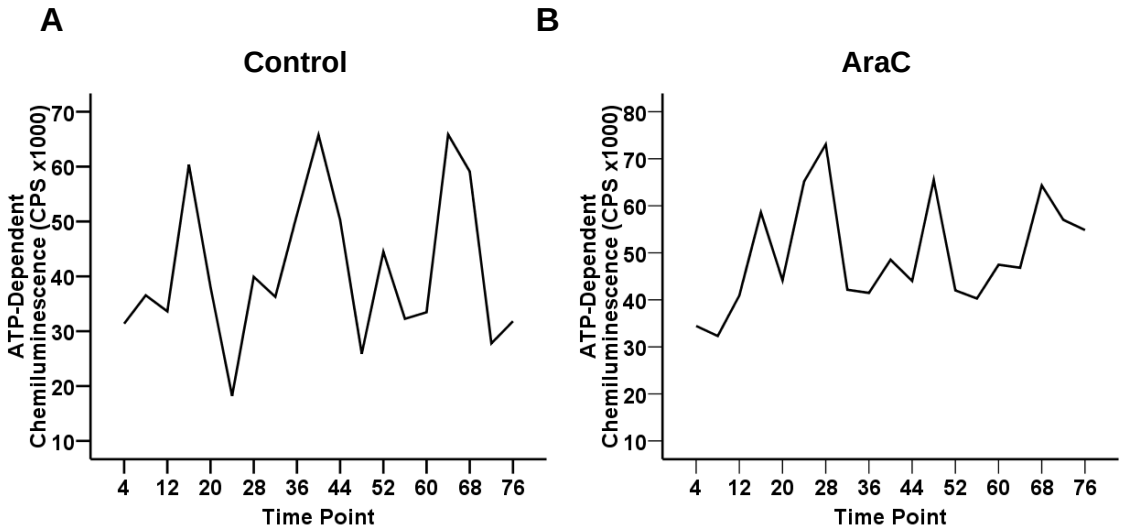

Fig. 11. Pharmacological disruption of the cell cycle with AraC did not affect rhythmic extracellular ATP accumulation. **A)** Single representative control SCN2.2 cell culture showed peaks in ATP accumulation in culture medium with approximately 24 hour periodicity (n=5). **B)** The addition of AraC, an anti-cancer drug used to inhibit cell division, did not significantly change the circadian ATP accumulation rhythm; extracellular ATP peaks of approximately 24 hours persist in the presence of the inhibitor.

rhythm, as ATP peaks with approximately 24 hour periodicity persisted in AraC-treated cultures (n=7).

To confirm that the SCN2.2 cell cycle was indeed being antagonized by AraC, SCN2.2 cell cultures were imaged for cell count and chromatin analysis using DAPI stain to mark cell nuclei. Addition of AraC to the cultures caused a significant decrease in overall cell count when compared to control untreated cultures ($p<0.05$; n=8) (Fig. 12). However, the ATP trough and ATP peak were still present in AraC-treated cultures, despite disruption of the cell cycle. Thus, cell cycle events were not mechanistically linked to generation of the rhythmic accumulation of ATP in culture medium; that is, extracellular ATP accumulation is not a byproduct of rhythmic cell division.

Clock-Controlled ATP accumulation Is Not Dependent on Rhythmic Cell Death

Both purinergic and mitochondrial calcium signaling have been implicated in cell death processes (Locovei et al., 2007; Hajnoczky et al., 2006). Increases in mitochondrial matrix calcium evoked by calcium mobilizing agonists play a fundamental role in the physiological control of cellular energy metabolism as well as apoptosis, because these calcium signals elicit cytochrome c release which activates caspases. Recently, cytochrome c gene regulation has been discovered to be circadian in rat SCN cells (Menger et al, 2005). I was interested in exploring the possible correlation between caspase activity and the SCN2.2 basal ATP accumulation rhythm. Therefore, I used a Pan-Caspase assay to fluorescently mark caspases at the time points associated with peaks and troughs of extracellular ATP accumulation. There was no correlation

between caspase activity and extracellular ATP accumulation levels (Fig. 13). The ratio of caspase-positive cells over the total number of cells determined by DAPI staining was not significantly different between the time points. Therefore, clock-controlled extracellular ATP accumulation was not a byproduct of rhythmic cell death.

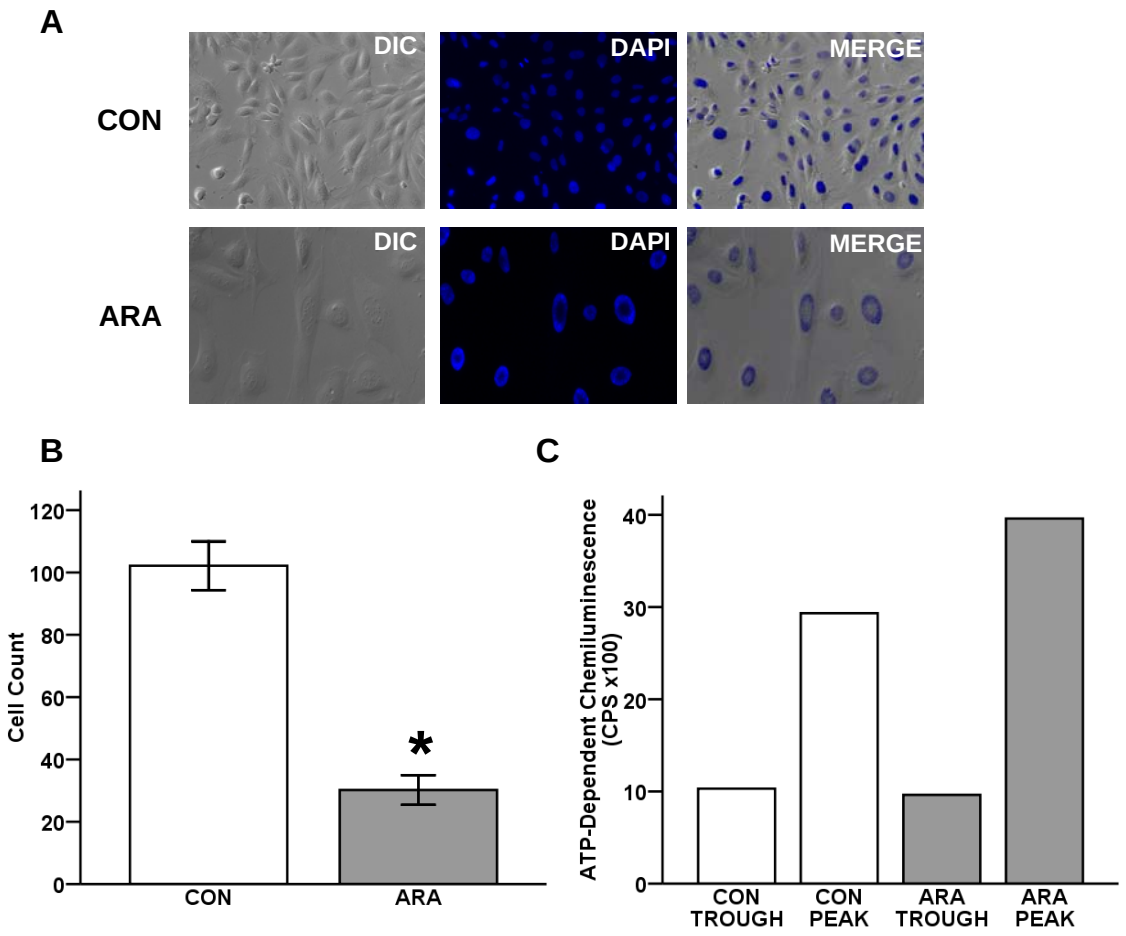

Fig. 12. Pharmacological disruption of the cell cycle with AraC affected cell number, but not rhythmic ATP accumulation. **A)** Representative images of control and AraC-treated (ARA) SCN2.2 cultures. (*From left to right*: 20X DIC, 20X DAPI, and 20X DIC/DAPI merged images). **B)** Addition of AraC inhibited cell division, leading to significantly reduced cell counts compared to control (*$p<0.05$; $n=8$). **C)** The ATP trough and ATP peak are present in both control and AraC-treated SCN2.2 cultures. Error bars represent 95% confidence intervals.

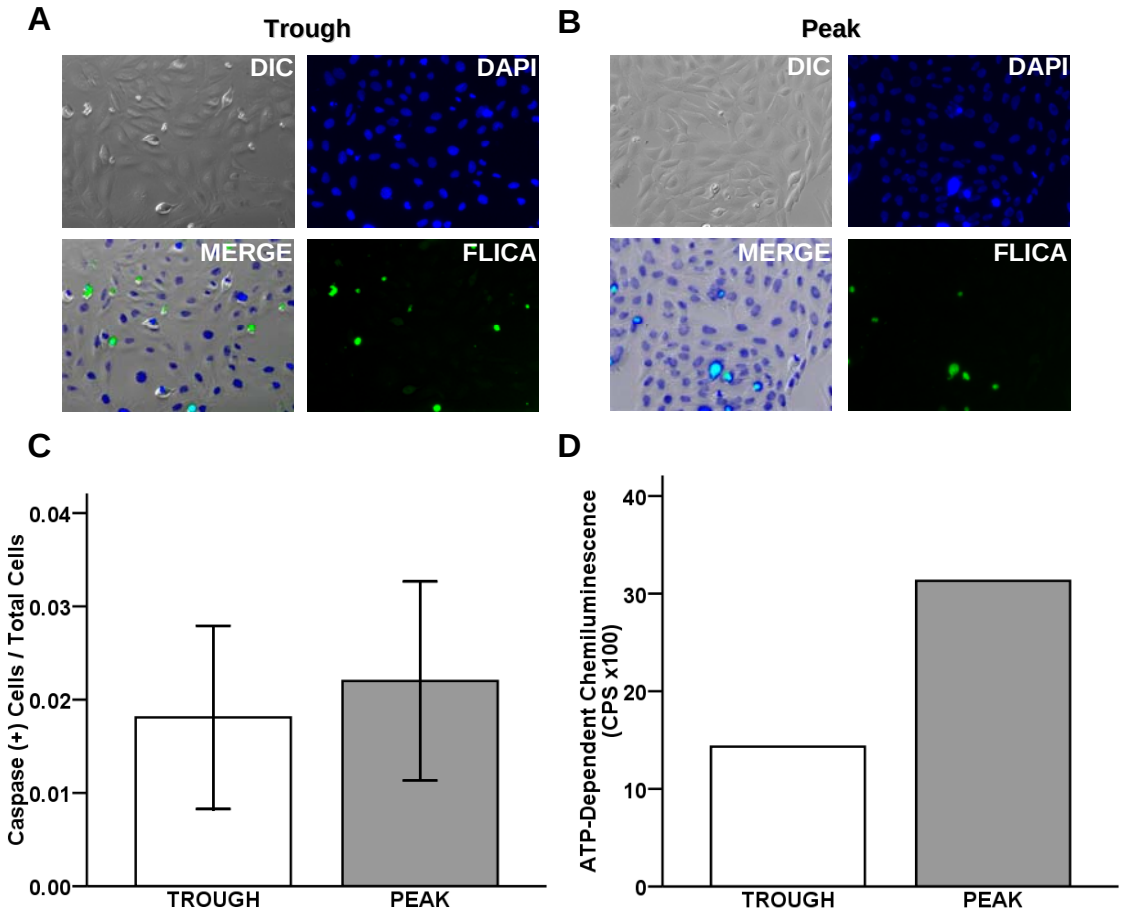

Fig. 13. Caspase activity did not correlate with cell count nor ATP levels. **A)** Representative images of SCN2.2 cultures on 2-well glass chamberslides at ATP trough. (*Clockwise from upper left*: 20X DIC, 20X DAPI, 20X FLICA, and 20X DIC/DAPI/FLICA merged images). **B)** Representative images of SCN2.2 cultures on 2-well glass chamberslides at ATP peak. (*Clockwise from upper left*: 20X DIC, 20X DAPI, 20X FLICA, and 20X DIC/DAPI/FLICA merged images). **C)** There was no difference in caspase activity between the ATP trough and the ATP peak ($p>.05$; $n=8$). **D)** The presence of an ATP trough and ATP peak was confirmed using the Luc/Luc assay. Error bars represent 95% confidence intervals.

CHAPTER IV

CONCLUSIONS

The circadian timing system regulates many aspects of an organism's behavior and physiology. In mammals, the part of the nervous system responsible for most circadian behavior can be found in the paired suprachiasmatic nuclei (SCN) of the hypothalamus. Importantly, when these nuclei are excised and maintained in brain slice culture, they continue to generate 24 hour rhythms in physiology and gene expression. The mechanism responsible for the generation of these rhythms is inherent to individual cells in the SCN (Welsh et al., 1995). The core molecular mechanism driving these cellular oscillations is a negative feedback loop operating at the level of transcription/translation (Reppert, 1998). Conceptually it is important to think of the circadian system as having three components: (1) a multicellular clock responsible for the generation of the daily rhythm, (2) input pathways by which the environment and other components of the nervous system provide information to the clock, and (3) output pathways by which the multicellular clock provides temporal information to a wide range of physiological and behavioral control centers. Nevertheless, the path by which environmental signals travel to the oscillatory mechanism and by which the oscillatory mechanism regulates other physiological processes clearly travels through the cell membrane. The circadian interplay between critical signaling processes is thought to be a widespread feature of circadian timing systems (Pearson et al., 2002; Rascher et al., 2001; Wijnen and Young, 2006). The present study implicates purinergic signaling at

the SCN cell membrane and intra/intercellular calcium signaling mechanisms as important elements of the SCN biological clock.

Characterization of Two ATP-Dependent Signaling Processes in SCN2.2 Cells

In the present study, varied approaches were utilized to confirm the existence of two independent ATP-dependent signaling processes in SCN2.2 cells: (1) clock-controlled ATP accumulation and (2) ATP-dependent stimulus-evoked gliotransmission. ATP accumulation in SCN2.2 cell cultures is rhythmic (Womac et al., 2009) and these results were confirmed in the present study. This clock-controlled ATP accumulation rhythm exhibited ATP levels of ~1 pM at its trough and ~5-10 nM at its peak time point in unstimulated cells. A circadian rhythm in resting cytosolic calcium levels was found to oscillate in antiphase to peaks and troughs in extracellular ATP accumulation, a finding perhaps inconsistent with a role for cytoplasmic calcium signaling in clock-controlled ATP release.

There are many areas of current interest in the rapidly expanding purinergic signaling field that are controversial and unresolved. One of these areas actively debated is the precise transport mechanism(s) involved in ATP release. There is compelling evidence for exocytotic vesicular release of ATP from the active zones of nerve terminals. Vesicular release of ATP is thought to mediate stimulus-evoked calcium-dependent purinergic signaling among astrocytes (Pangrsic et al., 2007). Still, the mechanisms for ATP release from nonneuronal cells remain controversial and various transport mechanisms have been proposed, including ATP-binding cassette (ABC)

transporters, connexin, or pannexin hemichannels or possibly plasmalemmal voltage-dependent anion and $P2X_7$ receptor channels (Bodin and Burnstock, 2001; Dahl and Locovei, 2006; De Vuyst et al., 2006; Lazarowski et al., 2003; Sabirov and Okada, 2005; Schwiebert and Zsembery, 2003; Spray et al., 2006).

In this model of ATP-dependent gliotransmission, released and extracellularly accumulated ATP binds to P2Y receptors on neighboring cells. This P2Y receptor activation leads to activation of an IP_3-dependent signaling mechanism and the elevation of resting calcium levels via release from intracellular ER stores (Charles et al., 1991). Elevation of calcium levels elicits a calcium-dependent vesicular release of ATP, which in turn evokes calcium responses in neighboring astrocytes, often generating an intercellular astrocytic calcium wave. In agreement with these studies, stimulation-evoked calcium waves among SCN astrocytes were significantly reduced by a P2Y receptor antagonist (PPADS). On the contrary, clock-controlled ATP accumulation was not affected by this P2Y antagonist; rather, the $P2X_7$ receptor antagonist, BBG, significantly increased this rhythmic ATP accumulation.

The $P2X_7$ receptor is unique among members of the ionotropic P2XR family because it has a long COOH terminus (~32 kDa) that provides sites for protein-protein interactions (Kim et al., 2001a; Kim et al., 2001b). The diversity of molecules found to interact with the $P2X_7$ receptor includes laminin, actin, actinin, integrins, phosphatidylinositol 4-kinase, and receptor protein phosphatase (Kim et al., 2001a); however, the functional significance of such associations remain unclear. Overall, caution must be used when interpreting the effects of BBG on clock-controlled ATP

signaling because the BBG-specific mechanisms of antagonizing $P2X_7$ receptor signaling are unresolved. With that said, some aspect of $P2X_7$ receptor signaling is likely involved in clock-controlled ATP accumulation. These experiments implicate two ATP-dependent signaling processes in SCN2.2 cell cultures: clock-controlled ATP accumulation and stimulation-evoked ATP accumulation. The existence of multiple purinergic signaling pathways may be needed, among other things, to regulate basal metabolic activities of the cell (clock-controlled ATP accumulation) or paracrine processes which vary depending on the characteristics of the stimulus (stimulation-evoked ATP accumulation).

ATP is converted to cyclic AMP (cAMP) by the enzyme adenylyl cyclase (AC). Recently, it was concluded that circadian pacemaking in mammals is sustained, and its canonical properties of amplitude, phase, and period are determined by a reciprocal interplay in which transcriptional and posttranslational loops drive rhythms of cAMP signaling, and that changes in cAMP signaling, in turn, regulate transcriptional cycles (O'Neill et al., 2008). Furthermore, the gene encoding the AC protein is rhythmically expressed in rat SCN cells (Menger et al., 2005). By utilizing pharmacological techniques to modulate AC activity, the effect of cAMP second messenger signaling on ATP accumulation was explored. Here, I demonstrated that cAMP levels affect clock-controlled ATP accumulation. Specifically, extracellular ATP accumulation was reduced by pharmacological elevations of AC activity. These findings indicate an interaction between cAMP and ATP signaling in the regulation of circadian pacemaker activity. Such an interaction is not too difficult to envision as ATP is the substrate used

in AC production of cAMP. In contrast to clock-controlled ATP accumulation, ATP-dependent calcium waves were not significantly affected by cAMP manipulation.

Calcium Signaling Mediates Clock-Controlled ATP Gliotransmission

Information about diverse physiological and developmental events is transmitted through changes in the cytosolic concentration of free calcium ions ($[Ca^{2+}]_i$), which often occur in oscillatory patterns of various amplitudes, frequencies, and durations (Allen and Schroeder, 2001; Berridge et al., 2003). Most calcium signals are brief, lasting from milliseconds to minutes. However, in both plants and animals, researchers have discovered slow $[Ca^{2+}]_i$ oscillations that spans an entire day (Johnson et al., 1995; Colwell, 2000). These circadian $[Ca^{2+}]_i$ oscillations are arguably some of the most stable $[Ca^{2+}]_i$ signals found in nature, in terms of their period and phase (Love et al., 2004). Here I have demonstrated that resting calcium levels oscillate in SCN2.2 cell cultures with cytosolic calcium concentration lowest when extracellular ATP accumulation is high. In turn, intracellular calcium is highest when ATP accumulation is low. Two separate calcium imaging studies, one using FURA-2 AM ratiometric analysis and the other using monochromatic FLUO-4 AM analysis, both demonstrated a 60% difference in resting calcium amplitude between the ATP peak and trough, an estimated 100 nM circadian calcium oscillation. My results with SCN2.2 cells are similar to SCN slice preparation studies, where circadian oscillations in resting calcium were observed with peak levels occurring during the day and trough levels during the night (Colwell, 2000). Furthermore, extracellular ATP accumulation is highest at night and lowest

during the day in the rat SCN *in vivo* (Womac et al., 2009). Since peak resting calcium levels in SCN2.2 cell cultures occurred when extracellular ATP accumulation was low, these results imply that cytoplasmic calcium signaling may not be an immediate regulator for clock-controlled ATP release.

Taken together, several lines of evidence from my studies support the idea that clock-controlled, extracellular ATP accumulation is regulated by cell signaling mechanisms that differ from those mediating stimulus-evoked ATP gliotransmission. First, elevations in cytoplasmic calcium trigger stimulated ATP gliotransmission, yet cytoplasmic calcium is lowest when clock-controlled ATP release is at peak amplitude. Second, stimulated ATP release involved in calcium wave propagation is P2Y receptor-dependent, whereas rhythmic ATP accumulation is not and may involve P2X receptor signaling. Third, and finally, manipulation of cellular cAMP levels affects clock-controlled ATP release in SCN2.2 cells, but does not alter the spread of ATP-dependent calcium waves.

While cytoplasmic calcium has been implicated in the spread of ATP-dependent astrocytic calcium waves (Charles et al., 1991), the present study set out to identify possible links between calcium signaling and the clock-controlled ATP accumulation rhythm. Recent advances have allowed intracellular free calcium concentrations to be examined not just at the level of a single cell, but within subcellular compartments and organelles (Rutter et al., 1998). For example, mitochondria function as calcium stores, dynamically participating in physiological calcium homeostasis (Nicholls, 1978; Griffiths and Rutter, 2009). Mitochondrial calcium has also been recognized as a

potential regulator of metabolism for 50 years, since Krebs discovered that it activated phosphorylase kinase in 1959 (Krebs et al., 1959). In the present study, the role of mitochondria in the circadian generation of this clock-controlled ATP accumulation phenomenon was considered. A RHOD-2 AM imaging study determined that when mitochondrial calcium ($[Ca^{2+}]_m$) is raised, clock-controlled ATP accumulation is simultaneously elevated. Further, when $[Ca^{2+}]_m$ is low, extracellular ATP accumulation is low. Therefore, in stark contrast to cytoplasmic calcium, mitochondrial calcium levels are directly correlated with clock-controlled ATP accumulation. The ATP accumulation peaks described in the present study as well as previous reports (Womac et al., 2009) have shown a very sharp rise and fall in amplitude. Interestingly, mitochondrial calcium accumulation in intact cells is similarly transient (Rizzuto et al., 1993). The unexpected high rate of this process in intact cells could, in principle, be due to a cytosolic (possibly clock-controlled) factor sharply increasing the affinity or maximal activity of the mitochondrial uniporter. The circadian clock in SCN2.2 cells influences mitochondrial energy transduction through the rhythmic expression of mitochondrial ATP synthase 8 (*mt-atp8*) and calcium transporting ATPase (*Atp2a3*) (Menger et al., 2005).

To further explore the role of mitochondrial calcium signaling in the generation of clock-controlled ATP accumulation, SCN2.2 cells were treated with Ru360, an inhibitor of the mitochondrial calcium uniporter that controls mitochondrial calcium uptake. ATP chemiluminescence at both trough and peak time points was abolished in Ru360-treated SCN2.2 cells. Wu et al. (2007) demonstrated that Ru360 significantly decreases basal ATP content in astrocytes. Therefore, sequestering of calcium in the

mitochondria is necessary for clock-controlled purinergic signaling in SCN2.2 cell cultures, since its disruption leads to the abolishment of clock-controlled ATP production. It is worth noting that intracellular ATP content in rat SCN cells *in vivo* is also rhythmic with peak levels detected at night (Yamazaki et al., 1994), the same phase relationship demonstrated for extracellular ATP rhythms in the rat SCN (Womac et al., 2009).

The endoplasmic reticulum (ER) represents another store of releasable calcium in the cell, with a free resting calcium concentration in the range of a few hundreds of μM, which is approximately 2-3 orders of magnitude higher than cytosolic levels (Hofer and Schulz, 1996). The resting calcium concentration in the ER is maintained as a balance of calcium uptake and calcium leak. Calcium uptake is mediated by pumps that belong to the sarcoplasmic-endoplasmic type of calcium ATPases (SERCA type). Using thapsigargin, an inhibitor of SERCA pumps, I demonstrated that while the spread of stimulus-evoked calcium waves is affected, the phase of the clock-controlled extracellular ATP accumulation rhythm is not. These data support the idea that SERCA pumps in SCN2.2 astrocytes are thapsigargin-sensitive and stimulation-evoked ATP-dependent gliotransmission in the SCN is dependent on SERCA pumps. However, at least the maintenance of clock-controlled ATP accumulation rhythms and their phase is apparently not dependent on ER calcium stores.

In the present study, cytoplasmic calcium was modulated with BAPTA-AM, a cytoplasmic calcium chelator to determine if the chelation of calcium disrupts the clock-controlled ATP accumulation rhythm. While a significant decrease of calcium wave

area was caused by chronic exposure of SCN2.2 cell cultures to BAPTA-AM, rhythms in calcium wave area and ATP accumulation persisted. Previous research in our lab has shown that clock-controlled extracellular ATP accumulation is correlated with significant rhythms in gliotransmission; that is, calcium wave areas were smaller when extracellular ATP was high and vice versa (Cox, 2007). In agreement with these studies, the Luc/Luc ATP chemiluminescence assay confirmed that endogenous extracellular ATP levels were greater when the calcium waves were small. Moreover, BAPTA-AM caused a dampening of the ATP accumulation rhythm. These results suggest the existence of an interaction between stimulus-evoked and clock-controlled gliotransmission, possibly manifested by a rhythm in the purinergic responsiveness of SCN2.2 cells (Cox, 2007). I have demonstrated here that ATP and calcium waves are both rhythmic and inversely correlated in cycling SCN2.2 cell cultures and that rhythm is not dramatically affected by chelation of cytoplasmic calcium for over 24 hours. Although a direct regulation of purinergic receptor gene expression by the clock is possible, or even likely, an equally plausible explanation is that accumulating extracellular ATP levels lead to the desensitization of purinergic receptors. This would create a state of reduced purinergic responsiveness at the peak of the ATP accumulation rhythm, when ATP-dependent calcium waves are reduced in magnitude.

At the ATP trough time point, cytoplasmic calcium levels are high and calcium wave area is the largest. Calcium wave propagation is dependent on vesicular release of ATP (Bowser and Khakh, 2007) and there is evidence that mitochondrial calcium buffering modulates ATP exocytosis (Giovannucci et al., 1999; Reyes and Parpura,

2008). This buffering regulates processes dependent on local cytoplasmic calcium concentration, particularly the flux of calcium through IP_3-gated channels of the ER and the channels mediating capacitative calcium influx through the plasma membrane (Duchen, 2000). With ATP production at the trough limited because mitochondrial calcium levels are lowered, there is more cytoplasmic calcium available to facilitate calcium-mediated fusion of ATP vesicles, refill ER calcium stores, and therefore enhance the spread of ATP-dependent calcium waves.

Consequences of Clock-Controlled ATP Signaling

Calcium signaling is integral to both clock-controlled ATP accumulation and stimulation-evoked ATP gliotransmission. Shifts in intracellular calcium handling between the cytoplasm and mitochondria may be mechanistically linked to clock-controlled ATP signaling in SCN cells. Therefore, I hypothesize that at late night in the rat SCN, when cytosolic calcium is low and extracellular ATP accumulation is high, calcium has been sequestered to the mitochondria, activating dehydrogenase enzymes to increase NADH and subsequently ATP production. At this time, stimulus-evoked ATP gliotransmission will be less effective. However, extracellular ATP will be converted by ectonucleotidases to adenosine. Elevated adenosine signaling, through specific classes of purinergic receptors, will impact primarily neighboring neurons, rather than glia. Since adenosine is known to be a regulator of sleep/wake homeostasis, this late night clock-controlled signaling is perfectly timed for a rat headed into its diurnal sleep phase. Furthermore, as mitochondrial calcium signaling is necessary to maintain the circadian

ATP rhythm, clock-controlled ATP gliotransmission is ideally positioned as a signaling link between metabolic homeostasis and sleep/wake regulation.

Along with the ATP accumulation and resting calcium rhythms, an important rhythm to consider is glucose uptake. SCN2.2 cells are capable of endogenously generating circadian rhythms in 2-deoxyglucose uptake (Earnest et al., 1999). Glucose demands change during a 24 hour light/dark cycle and glucose metabolism is modulated by the SCN in a rhythmic fashion, independent of the SCN's strong influence on food intake. It was recently demonstrated that 2-DG uptake in SCN2.2 cells coincides with peak time points of ATP accumulation (Cox, 2007). The uptake of 2-NBDG, a fluorescent glucose analog, was higher at the ATP peak time point in the clock-controlled ATP rhythm, as compared to the ATP trough time point. Thus, ATP appears to accumulate in the extracellular medium of SCN2.2 cell cultures during the same time that these cultures experience high glucose uptake. Again, these observations support a role for ATP signaling in the clock-control of brain metabolism.

As described earlier, ATP levels in the rat SCN *in vivo* remained low throughout the daytime and the first half of the night, rapidly increasing near the middle of the dark phase is phase-locked to circadian oscillations in glucose uptake. However, SCN resting cytosolic calcium oscillations are locked in antiphase (Fig. 14). With a recent revelation that the circadian cycle is linked directly to the metabolic cycle, the integration of clock-controlled ATP signaling and metabolism begs further exploration (Rutter et al., 2002). First, transcriptional profiling studies indicate that the expression of genes involved in ATP signaling and metabolic pathways is similarly clock-controlled in SCN2.2 cell

cultures and the SCN *in vivo* (Menger et al., 2005; Panda et al., 2002). The circadian clock in SCN2.2 cells influences mitochondrial energy transduction through the rhythmic expression of mitochondrial ATP synthase 8 (*mt-atp8*) and calcium transporting ATPase (*Atp2a3*), and affects glucose metabolism by regulating energy transduction through the rhythmic expression of malic enzyme 1 (*Me1*), hexokinase 2 (*Hk2*), and glyoxylate reductase/hydroxypyruvate reductase, an enzyme that mediates the conversion of serine to glucose. Second, cellular content of ATP (Yamazaki et al., 1994) and cAMP (Prosser and Gillette, 1989) oscillate on a circadian basis and adenylyl cyclase, type III (*Adycy3*) is rhythmically expressed in the rat SCN (Menger et al., 2005) (Fig. 15).

Clock-Controlled ATP Gliotransmission Is Neither a Byproduct of Cell Cycle Nor Cell Death

Although normally quiescent, astrocytes in the adult nervous system have the ability to proliferate. Extracellular ATP can enhance the mitogenic activity of polypeptide growth factors. In astrocytes, it has been shown that extracellular ATP markedly increases FGF2-induced mitogenesis (Neary et al, 2005). However, little is known about the interactive effects of extracellular nucleotides and growth factors on cell cycle regulatory mechanisms. Data from the present study indicate that the clock-controlled ATP rhythm is not a byproduct of rhythmic cell division. Arabinosylcytosine (AraC), an anti-cancer drug that affects cells when they are undergoing cell division, did not affect clock-controlled ATP accumulation, which maintained a circadian rhythm of

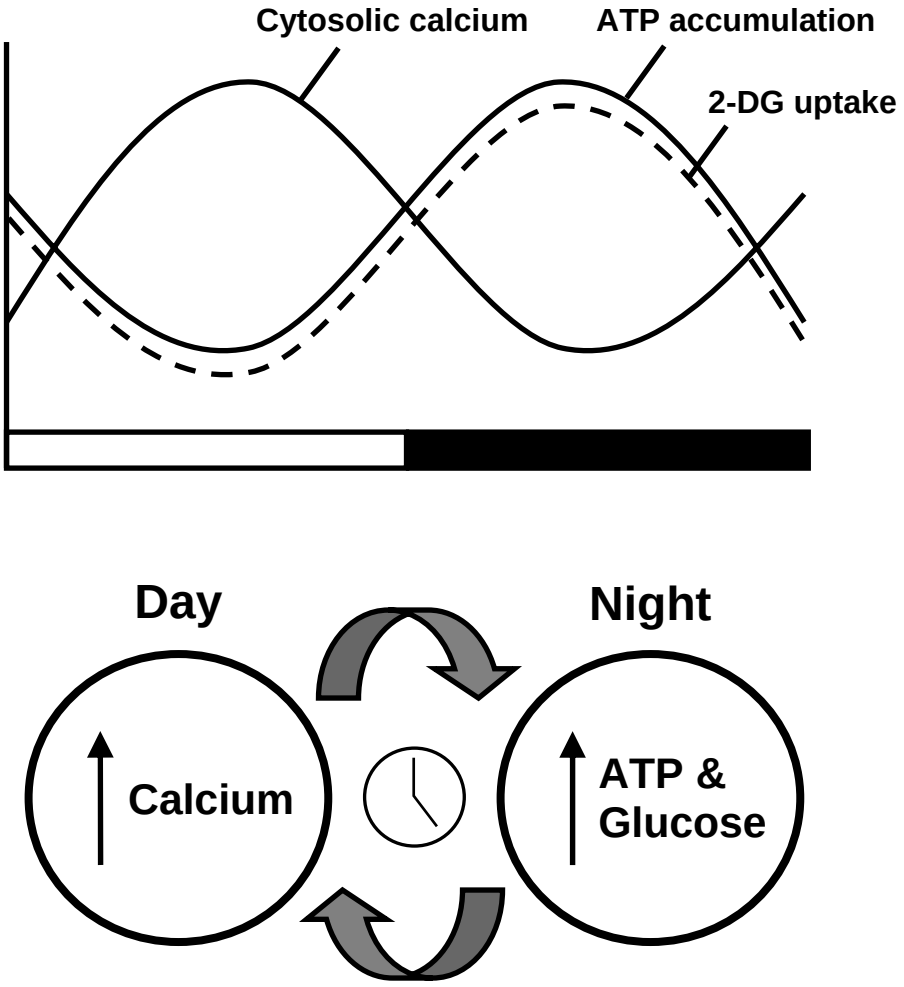

Fig. 14. Proposed temporal relationship between resting cytosolic calcium and ATP accumulation in the rat SCN. Intercellular communication among SCN cells can be functionally shifted to accommodate daytime versus nighttime physiological needs. In the middle of the night, clock-controlled ATP accumulation and glucose utilization is highest when this nocturnal animal is most active. Resting calcium levels peak during the diurnal rest phase, in precise antiphase to the ATP and 2-DG rhythms.

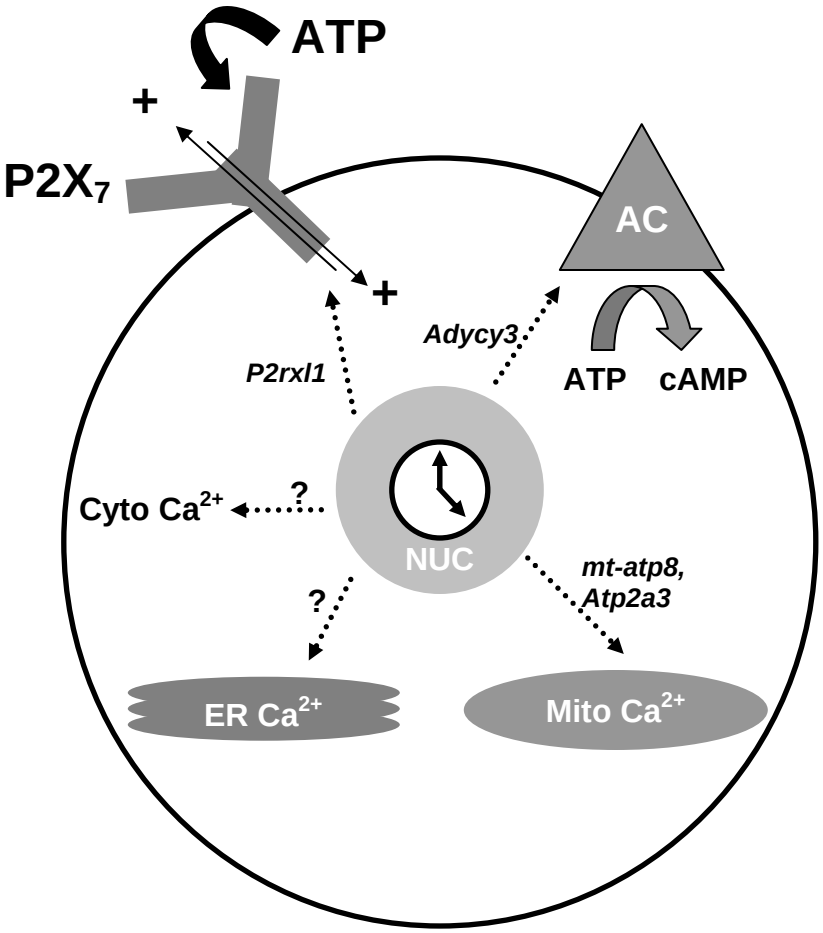

Fig. 15. The circadian clock in SCN2.2 cells influences components of the clock-controlled ATP accumulation rhythm through rhythmic gene expression. The circadian clock influences energy transduction through the rhythmic expression of mitochondrial ATP synthase 8 (*mt-atp8*) and calcium transporting ATPase (*Atp2a3*), purinergic signaling through the expression of P2X-like 1 receptor (*P2rxl1*), and cAMP activity through rhythmic expression of adenylyl cyclase type III (*Adcy3*).

approximately 24 hours. Of additional concern, calcium signal propagation to the mitochondria can also initiate cell death through the opening of the permeability transition pore (PTP), which occurs when the ER calcium release is enhanced or is coincident with sensitization of the PTP (Hajnoczky et al., 2006). Several pro-apoptotic factors, including members of the Bcl-2 family proteins and reactive oxygen species (ROS) regulate the calcium sensitivity of both the calcium-release channels in the ER and the PTP in the mitochondria (Chen et al., 2004; Zorov et al., 2000). In the present study, I demonstrated that caspase-7 activity is not correlated with ATP accumulation rhythms in SCN2.2 cell cultures, where only 2% of all cells were apoptotic at a given time point. Therefore, the clock-controlled extracellular ATP accumulation rhythm is not a byproduct of rhythmic cell cycle or cell death in this culture system.

Characterization of Rhythmic Physiological Outputs in SCN2.2 Cultures

The present study involved the development of standardized protocols to characterize ATP accumulation and calcium imaging in mitochondria and cytoplasm during both clock-controlled and stimulus-evoked ATP accumulation gliotransmission in SCN2.2 cell cultures. The quantification of ATP accumulation in live cells over a time series of multiple circadian cycles allowed the establishment of the ATP accumulation rhythms to be examined together with critical cell pathways. The further development of the SCN2.2 culture model of the mammalian pacemaker, with verifiable time points for the study of physiological outputs, establishes a stable tool for *in vitro* analysis of clock-controlled neural signaling.

Summary

The current study has identified two disparate mechanisms of ATP signaling: clock-controlled ATP accumulation and stimulus-evoked ATP-dependent gliotransmission. I argue that the clock-controlled ATP rhythm is mechanistically linked to the brain's metabolic function, such that at late night, mitochondrial calcium levels are raised, leading to greater production of ATP, perhaps as a signal for the approaching sleep phase. Though stimulus-evoked ATP gliotransmission may not be directly regulated by clock-controlled processes, there are likely certain components of its signaling cascade imposed upon by the clock.

In summary, clock-controlled ATP accumulation and stimulus-evoked ATP are disparate signaling mechanisms in SCN2.2 cultures and regulated by different purinergic components. Calcium signaling is important to the function of both these two purinergic signaling processes. Clock-controlled ATP accumulation is not a byproduct of cell cycle or cell death. It will be important in future studies to determine the specific functions and mechanisms of circadian ATP signaling, as such findings will be crucial to a complete understanding of circadian timekeeping by the SCN.

REFERENCES

Abbracchio MP, Burnstock G (1994) Purinoceptors: are there families of P2X and P2Y purinoceptors? Pharmacol Ther 64:445-475.

Abbracchio MP, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Miras-Portugal MT, King BF, Gachet C, Jacobson KA, Weisman GA, Burnstock G (2003) Characterization of the UDP-glucose receptor (re-named here the P2Y14 receptor) adds diversity to the P2Y receptor family. Trends Pharmacol Sci 24:52-55.

Abbracchio MP, Burnstock G, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Knight GE, Fumagalli M, Gachet C, Jacobson KA, Weisman GA (2006) International union of pharmacology LVIII: Update on the P2Y G protein-coupled nucleotide receptors: From molecular mechanisms and pathophysiology to therapy. Pharmacol Rev 58:281-341.

Allen G, Rappe J, Earnest DJ, Cassone VM (2001) Oscillating on borrowed time: diffusible signals from immortalized suprachiasmatic nucleus cells regulate circadian rhythmicity in cultured fibroblasts. J Neurosci 21:7937-7943.

Allen GJ, Schroeder JI (2001) Combining genetics and cell biology to crack the code of plant cell calcium signaling. Sci STKE 2001:RE13.

Araque A, Parpura V, Sanzgiri RP, Haydon PG (1999) Tripartite synapses: glia, the unacknowledged partner. Trends Neurosci 22:208-215.

Araque A, Perea G (2004) Glial modulation of synaptic transmission in culture. Glia 47:241-248.

Arcuino G, Lin JH, Takano T, Liu C, Jiang L, Gao Q, Kang J, Nedergaard M (2002) Intercellular calcium signaling mediated by point-source burst release of ATP. Proc Natl Acad Sci USA 99:9840-9845.

Baran I (1996) Calcium and cell cycle progression: possible effects of external perturbations on cell proliferation. Biophys J 70:1198-1213.

Bennett MV, Contreras JE, Bukauskas FF, Saez JC (2003) New roles for astrocytes: gap junction hemichannels have something to communicate. Trends Neurosci 26:610-617.

Berridge MJ, Bootman MD, Roderick HL (2003) Calcium signalling: dynamics, homeostasis and remodelling. Nat Rev Mol Cell Biol 4:517-529.

Bodin P, Burnstock G (2001) Purinergic signalling: ATP release. Neurochem Res 26:959-969.

Bowser DN, Khakh BS (2007) Vesicular ATP is the predominant cause of intercellular calcium waves in astrocytes. J Gen Physiol 129:485-491.

Burnstock G, Cocks T, Kasakov L, Wong HK (1978) Direct evidence for ATP release from non-adrenergic, non-cholinergic (purinergic) nerves in guinea-pig taenia-coli and bladder. Eur J Pharmacol 49:145-149.

Burnstock G, Knight GE (2004) Cellular distribution and functions of P2 receptor subtypes in different systems. Int Rev Cytol 240:31-304.

Charles AC, Merrill JE, Dirksen ER, Sanderson MJ (1991) Intercellular signaling in glial cells: calcium waves and oscillations in response to mechanical stimulation and glutamate. Neuron 6:983-992.

Chen R, Valencia I, Zhong F, McColl KS, Roderick HL, Bootman MD, Berridge MJ, Conway SJ, Holmes AB, Mignery GA, Velez P, Distelhorst CW (2004) Bcl-2 functionally interacts with inositol 1,4,5-trisphosphate receptors to regulate calcium release from the ER in response to inositol 1,4,5-trisphosphate. J Cell Biol 166:193-203.

Colwell CS (2000) Circadian modulation of calcium levels in cells in the suprachiasmatic nucleus. Eur J Neurosci 12:571-576.

Cotrina ML, Lin JH, Alves-Rodrigues A, Liu S, Li J, Azmi-Ghadimi H, Kang J, Naus CC, Nedergaard M (1998) Connexins regulate calcium signaling by controlling ATP release. Proc Natl Acad Sci USA 95:15735-15740.

Cox KY (2007) Melatonin modulates intercellular communication among immortalized rat suprachiasmatic nucleus cells. M.S. thesis. College Station: Texas A&M University.

Dahl G, Locovei S (2006) Pannexin: to gap or not to gap, is that a question? IUBMB Life 58:409-419.

Darby M, Kuzmiski JB, Panenka W, Feighan D, MacVicar BA (2003) ATP released from astrocytes during swelling activates chloride channels. J Neurophysiol 89:1870-1877.

De Vuyst E, Decrock E, Cabooter L, Dubyak GR, Naus CC, Evans WH, Leybaert L (2006) Intracellular calcium changes trigger connexin 32 hemichannel opening. Embo Journal 25:34-44.

Dermietzel R, Traub O, Hwang TK, Beyer E, Bennett MV, Spray DC, Willecke K (1989) Differential expression of three gap junction proteins in developing and mature brain tissues. Proc Natl Acad Sci USA 86:10148-10152.

Dermietzel R, Hertberg EL, Kessler JA, Spray DC (1991) Gap junctions between cultured astrocytes: immunocytochemical, molecular, and electrophysiological analysis. J Neurosci 11:1421-1432.

Dermietzel R, Spray DC (1993) Gap junctions in the brain: where, what type, how many and why? Trends Neurosci 16:186-192.

Duchen MR (2000) Mitochondria and calcium: from cell signalling to cell death. J Physiol 529 Pt 1:57-68.

Earnest DJ, Liang FQ, Ratcliff M, Cassone VM (1999) Immortal time: circadian clock properties of rat suprachiasmatic cell lines. Science 283:693-695.

Ebihara L (2003) New roles for connexons. News Physiol Sci 18:100-103.

Fields RD, Burnstock G (2006) Purinergic signalling in neuron-glia interactions. Nat Rev Neurosci 7:423-436.

Fredholm BB, AP IJ, Jacobson KA, Klotz KN, Linden J (2001) International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. Pharmacol Rev 53:527-552.

Fumagalli M, Brambilla R, D'Ambrosi N, Volonte C, Matteoli M, Verderio C, Abbracchio MP (2003) Nucleotide-mediated calcium signaling in rat cortical astrocytes: Role of P2X and P2Y receptors. Glia 43:218-203.

Gery S, Komatsu N, Baldjyan L, Yu A, Koo D, Koeffler HP (2006) The circadian gene per1 plays an important role in cell growth and DNA damage control in human cancer cells. Mol Cell 22:375-382.

Gillette MU, Reppert SM (1987) The hypothalamic suprachiasmatic nuclei: circadian patterns of vasopressin secretion and neuronal activity *in vitro*. Brain Res Bull 19:135-139.

Giovannucci DR, Hlubek MD, Stuenkel EL (1999) Mitochondria regulate the Ca^{2+}-exocytosis relationship of bovine adrenal chromaffin cells. J Neurosci 19:9261-9270.

Griffiths EJ, Rutter GA (2009) Mitochondrial calcium as a key regulator of mitochondrial ATP production in mammalian cells. Biochim Biophys Acta.

Guthrie PB, Knappenberger J, Segal M, Bennett MV, Charles AC, Kater SB (1999) ATP released from astrocytes mediates glial calcium waves. J Neurosci 19:520-528.

Hajnoczky G, Csordas G, Das S, Garcia-Perez C, Saotome M, Sinha Roy S, Yi M (2006) Mitochondrial calcium signalling and cell death: approaches for assessing the role of mitochondrial Ca^{2+} uptake in apoptosis. Cell Calcium 40:553-560.

Halassa MM, Florian C, Fellin T, Munoz JR, Lee SY, Abel T, Haydon PG, Frank MG (2009) Astrocytic modulation of sleep homeostasis and cognitive consequences of sleep loss. Neuron 61:156-157.

Haydon PG (2001) GLIA: listening and talking to the synapse. Nat Rev Neurosci 2:185-193.

Ho C, Hicks J, Salter MW (1995) A novel P2-purinoceptor expressed by a subpopulation of astrocytes from the dorsal spinal cord of the rat. Br J Pharmacol 116:2909-2918.

Hofer AM, Schulz I (1996) Quantification of intraluminal free [Ca] in the agonist-sensitive internal calcium store using compartmentalized fluorescent indicators: some considerations. Cell Calcium 20:235-242.

Jacobson KA, Gao ZG (2006) Adenosine receptors as therapeutic targets. Nat Rev Drug Discov 5:247-264.

Johnson CH, Knight MR, Kondo T, Masson P, Sedbrook J, Haley A, Trewavas A (1995) Circadian oscillations of cytosolic and chloroplastic free calcium in plants. Science 269:1863-1865.

Kalsbeek A, Palm IF, La Fleur SE, Scheer FA, Perreau-Lenz S, Ruiter M, Kreier F, Cailotto C, Buijs RM (2006) SCN outputs and the hypothalamic balance of life. J Biol Rhythms 21:458-469.

Kim M, Jiang LH, Wilson HL, North RA, Surprenant A (2001a) Proteomic and functional evidence for a P2X7 receptor signalling complex. EMBO J 20:6347-6358.

Kim M, Spelta V, Sim J, North RA, Surprenant A (2001b) Differential assembly of rat purinergic P2X7 receptor in immune cells of the brain and periphery. J Biol Chem 276:23262-23267.

Klein DC, Moore RY, Reppert SM (1991) Suprachiasmatic nucleus: the mind's clock. New York: Oxford UP.

Krebs EG, Graves DJ, Fischer EH (1959) Factors affecting the activity of muscle phosphorylase b kinase. J Biol Chem 234:2867-2873.

Kuhlman SJ, McMahon DG (2006) Encoding the ins and outs of circadian pacemaking. J Biol Rhythms 21:470-481.

Lazarowski ER, Boucher RC, Harden TK (2003) Mechanisms of release of nucleotides and integration of their action as P2X- and P2Y-receptor activating molecules. Mol Pharmacol 64:785-795.

Locovei S, Scemes E, Qiu F, Spray DC, Dahl G (2007) Pannexin1 is part of the pore forming unit of the P2X(7) receptor death complex. FEBS Lett 581:483-488.

Love J, Dodd AN, Webb AA (2004) Circadian and diurnal calcium oscillations encode photoperiodic information in Arabidopsis. Plant Cell 16:956-966.

Marcussen M, Larsen PJ (1996) Cell cycle-dependent regulation of cellular ATP concentration, and depolymerization of the interphase microtubular network induced by elevated cellular ATP concentration in whole fibroblasts. Cell Motil Cytoskeleton 35:94-99.

Menger GJ, Lu K, Thomas T, Cassone VM, Earnest DJ (2005) Circadian profiling of the transcriptome in immortalized rat SCN cells. Physiol Genomics 21:370-381.

Moore RY, Eichler VB (1972) Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in rat. Brain Res 42:201-206.

Moore RY, Speh JC, Leak RK (2002) Suprachiasmatic nucleus organization. Cell and Tissue Research 309:89-98.

Neary JT, Kang Y, Shi YF (2005) Cell cycle regulation of astrocytes by extracellular nucleotides and fibroblast growth factor-2. Pur Sig 1:329-336.

Nicholls DG (1978) The regulation of extramitochondrial free calcium ion concentration by rat liver mitochondria. Biochem J 176:463-474.

North RA (2002) Molecular physiology of P2X receptors. Physiol Rev 82:1013-1067.

O'Neill JS, Maywood ES, Chesham JE, Takahashi JS, Hastings MH (2008) cAMP-dependent signaling as a core component of the mammalian circadian pacemaker. Science 320:949-953.

Panatier A, Oliet SH (2006) Neuron-glia interactions in the hypothalamus. Neuron Glia Biol 2:51-58.

Panda S, Antoch MP, Miller BH, Su AI, Schook AB, Straume M, Schultz PG, Kay SA, Takahashi JS, Hogenesch JB (2002) Coordinated transcription of key pathways in the mouse by the circadian clock. Cell 109:307-320.

Pangrsic T, Potokar M, Stenovec M, Kreft M, Fabbretti E, Nistri A, Pryazhnikov E, Khiroug L, Giniatullin R, Zorec R (2007) Exocytotic release of ATP from cultured astrocytes. J Biol Chem 282:28749-28758.

Parri HR, Gould TM, Crunelli V (2001) Spontaneous astrocytic Ca^{2+} oscillations in situ drive NMDAR-mediated neuronal excitation. Nat Neurosci 4:803-812.

Pascual O, Casper KB, Kubera C, Zhang J, Revilla-Sanchez R, Sul JY, Takano H, Moss SJ, McCarthy K, Haydon PG (2005) Astrocytic purinergic signaling coordinates synaptic networks. Science 310:113-116.

Pasti L, Volterra A, Pozzan T, Carmignoto G (1997) Intracellular calcium oscillations in astrocytes: a highly plastic, bidirectional form of communication between neurons and astrocytes in situ. J Neurosci 17:7817-7830.

Pearson R, Catsicas M, Becker D, Mobbs P (2002) Purinergic and muscarinic modulation of the cell cycle and calcium signaling in the chick retinal ventricular zone. J Neurosci 22:7569-7579.

Pennartz CM, de Jeu MT, Bos NP, Schaap J, Geurtsen AM (2002) Diurnal modulation of pacemaker potentials and calcium current in the mammalian circadian clock. Nature 416:286-290.

Peters JL, Cassone VM, Zoran MJ (2005) Melatonin modulates intercellular communication among cultured chick astrocytes. Brain Res 1031:10-19.

Porkka-Heiskanen T (1999) Adenosine in sleep and wakefulness. Ann Med 31:125-129.

Porter JT, McCarthy KD (1996) Hippocampal astrocytes in situ respond to glutamate released from synaptic terminals. J Neurosci 16:5073-5081.

Prosser RA, Gillette MU (1989) The mammalian circadian clock in the suprachiasmatic nuclei is reset *in vitro* by cAMP. J Neurosci 9:1073-1081.

Queiroz G, Meyer DK, Meyer A, Starke K, von Kugelgen I (1999) A study of the mechanism of the release of ATP from rat cortical astroglial cells evoked by activation of glutamate receptors. Neuroscience 91:1171-1181.

Ralph MR, Foster RG, Davis FC, Menaker M (1990) Transplanted suprachiasmatic nucleus determines circadian period. Science 247:975-978.

Rascher U, Hutt MT, Siebke K, Osmond B, Beck F, Luttge U (2001) Spatiotemporal variation of metabolism in a plant circadian rhythm: the biological clock as an assembly of coupled individual oscillators. PNAS 98:11801-11805.

Reppert SM (1998) A clockwork explosion! Neuron 21:1-4.

Reyes RC, Parpura V (2008) Mitochondria modulate Ca2+-dependent glutamate release from rat cortical astrocytes. J Neurosci 28:9682-9691.

Rizzuto R, Brini M, Murgia M, Pozzan T (1993) Microdomains with high calcium close to IP_3-sensitive channels that are sensed by neighboring mitochondria. Science 262:744-747.

Rutter GA, Fasolato C, Rizzuto R (1998) Calcium and organelles: a two-sided story. Biochem Biophys Res Comm 253:549-557.

Rutter J, Reick M, McKnight SL (2002) Metabolism and the control of circadian rhythms. Annu Rev Biochem 71:307-331.

Sabirov RZ, Okada Y (2005) ATP release via anion channels. Purinergic Signal 1:311-328.

Scemes E, Giaume C (2006) Astrocyte calcium waves: what they are and what they do. Glia 54:716-725.

Schwiebert EM, Zsembery A (2003) Extracellular ATP as a signaling molecule for epithelial cells. Biochim Biophys Acta 1615:7-32.

Spray DC, Ye ZC, Ransom BR (2006) Functional connexin "hemichannels": a critical appraisal. Glia 54:758-773.

Suadicani SO, De Pina-Benabou MH, Urban-Maldonado M, Spray DC, Scemes E (2003) Acute downregulation of Cx43 alters P2Y receptor expression levels in mouse spinal cord astrocytes. Glia 42:160-171.

Suadicani SO, Brosnan CF, Scemes E (2006) P2X7 receptors mediate ATP release and amplification of astrocytic intercellular Ca^{2+} signaling. J Neurosci 26:1378-1385.

Sujino M, Masumoto K, Yamaguchi S, van der Horst GTJ, Okamura H, Inouye SIT (2003) Suprachiasmatic nucleus grafts restore circadian behavioral rhythms of genetically arrhythmic mice. Current Biology 13:664-668.

Volterra A, Magistretti PJ, Haydon PG (2002) The tripartite synapse: glia in synaptic transmission. New York: Oxford University Press.

Volterra A, Meldolesi J (2005) Astrocytes, from brain glue to communication elements: the revolution continues. Nat Rev Neurosci 6:626-640.

Welsh DK, Logothetis DE, Meister M, Reppert SM (1995) Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms. Neuron 14:697-706.

Wijnen H, Young MW (2006) Interplay of circadian clocks and metabolic rhythms. Annu Rev Genet 40:409-448.

Wink MR, Braganhol E, Tamajusuku AS, Lenz G, Zerbini LF, Libermann TA, Sevigny J, Battastini AM, Robson SC (2006) Nucleoside triphosphate diphosphohydrolase-2 (NTPDase2/CD39L1) is the dominant ectonucleotidase expressed by rat astrocytes. Neuroscience 138:421-432.

Womac AD, Burkeen JF, Neuendorff N, Zoran MJ, Earnest DJ (2009) Circadian rhythms of extracellular ATP accumulation in SCN cells and cultured astrocytes. Eur J Neurosci:In submission.

Wu J, Holstein JD, Upadhyay G, Lin DT, Conway S, Muller E, Lechleiter JD (2007) Purinergic receptor-stimulated IP3-mediated Ca2+ release enhances neuroprotection by increasing astrocyte mitochondrial metabolism during aging. J Neurosci 27:6510-6520.

Yamazaki S, Ishida Y, Inouye S (1994) Circadian rhythms of adenosine triphosphate contents in the suprachiasmatic nucleus, anterior hypothalamic area and caudate putamen of the rat--negative correlation with electrical activity. Brain Res 664:237-240.

Yamazaki S, Kerbeshian MC, Hocker CG, Block GD, Menaker M (1998) Rhythmic properties of the hamster suprachiasmatic nucleus *in vivo*. J Neurosci 18:10709-10723.

Zimmermann H (2000) Extracellular metabolism of ATP and other nucleotides. Naunyn Schmiedebergs Arch Pharmacol 362:299-309.

Zorov DB, Filburn CR, Klotz LO, Zweier JL, Sollott SJ (2000) Reactive oxygen species (ROS)-induced ROS release: a new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes. J Exp Med 192:1001-1014.

VITA

Name:	Jeffrey Franklin Burkeen
Address:	Department of Biology, Texas A&M University, College Station, TX 77843-3258
Email Address:	jburkeen@gmail.com
Education:	B.S., Biology, University of Notre Dame, 2005 M.S., Biology, Texas A&M University, 2009